Award Number W81XWH-15-1-0002

TITLE:

A New Ultra-Small Volume Fluid for Far-Forward, Non-Compressible Hemorrhage and Traumatic Brain Injury

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REPORT DATE: January 2016

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
January 2016	Final	1 Nov 2014 to 31 Oct 2015

4. TITLE AND SUBTITLE
A New Ultra-Small Volume Fluid for Far-Forward, Non-Compressible Hemorrhage and Traumatic Brain Injury

5a. CONTRACT NUMBER
W81XWH-15-1-0002.

5b. GRANT NUMBER
SO13004

5c. PROGRAM ELEMENT NUMBER

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT (200 words)
Noncompressible truncal hemorrhage is the leading cause of potentially survivable trauma in far-forward combat environments, and no effective therapy exists. Hemorrhage combined with traumatic brain injury (TBI) is particularly lethal. Our aim was to examine the effect of small-volume 3% NaCl adenosine, lidocaine and Mg^{2+} (ALM) bolus and 0.9% NaCl ALM 'drip' (3-4 hour) on resuscitation, cardiac function, hemostasis and survivablity in three rat models of: 1) hepatic hemorrhage (60% resection) and shock, 2) mild-to-moderate TBI, and 3) combined TBI and hepatic hemorrhage. In the first model, ALM therapy reduced uncontrolled blood loss by up to 60% and improved blood flow to the gut and kidney, and Hextend administered according to TCCC guidelines promoted internal bleeding, organ failure and early death. ALM's ability to significantly reduce blood loss may arise from its unique ability to improve cardiac function, correct coagulopathy, blunt systemic inflammation and improve tissue oxygenation. In the second study, ALM treatment protected against secondary brain injury and improved cardiac function following TBI, and in the third lethal model of TBI and hemorrhage, ALM therapy resulted in 50% survivability and 50% less internal blood loss compared to 100% mortality in 3% NaCl controls. Small-volume ALM fluid therapy may have wide applications for SOF medics/corpsman to improve warfighter survivability in far-forward environments.

15. SUBJECT TERMS
Small-volume, resuscitation, hypotensive, non-compressible, truncal, hemorrhage, TBI, coagulopathy, inflammation, ALM, adenosine, lidocaine, magnesium, ROTEM, cardiac, neurogenic, multiple-organ failure, hextend, fluid therapy

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	56	19b. TELEPHONE NUMBER *(include area code)*

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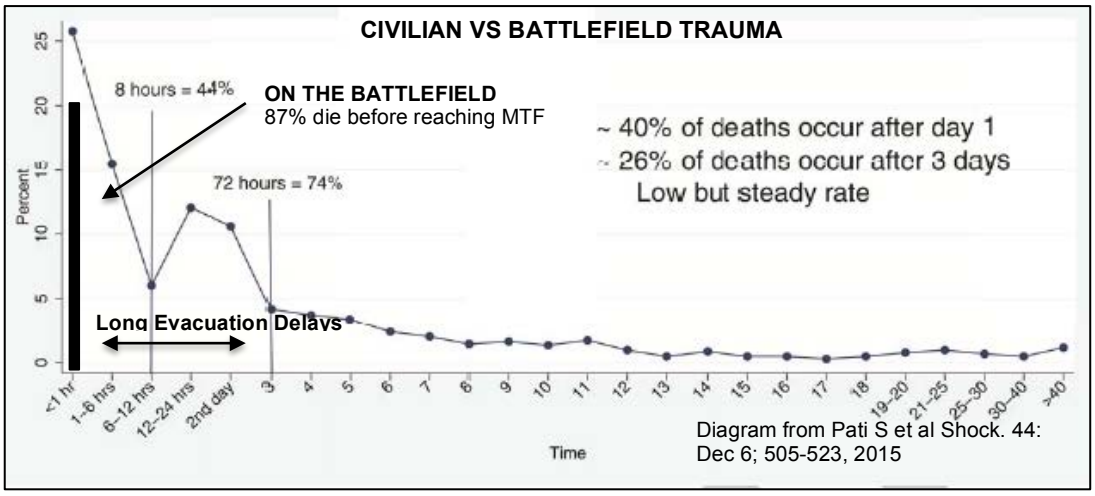
1. INTRODUCTION:

Subject: Hemorrhage with or without traumatic brain injury (TBI) is a major cause of death on the battlefield. Over 30 years ago, Col. Ronald Bellamy reported in his landmark article *The Causes of Death in Conventional Land Warfare*: "For every casualty who dies of wounds in a medical treatment facility (MTF), as many as 9 have already died". Bellamy was so concerned that he challenged the entire field stating: "there must be renewed emphasis on combat casualty care, with special attention to the management of hemorrhage on the battlefield" [Bellamy, 1984 #2647]. Despite extraordinary advances in hemorrhage control [Butler, 2014 #4057], the "Bellamy challenge" regarding non-compressible blood loss remains wide open [Dobson, 2014 #3143].

A 2012 US Joint Trauma System study reported that 87.3% of *combat deaths* in the Iraq and Afghanistan wars occurred *before* the casualty reached an MTF *(*4,596 deaths) [Eastridge, 2012 #3391], and 91% of these were from hemorrhage with 67% truncal (non-compressible), 19% junctional and 14% peripheral-extremity [Eastridge, 2012 #3391]. It was also estimated that 24.3% of these pre-MTF deaths were potentially survivable (976 lives). Traumatic brain injury (TBI) accompanying hemorrhage is also a significant contributor to battlefield deaths and morbidity. TBI is responsible for 20-25% of battle-incurred injuries, and for those suspected of TBI that reach a surgical ward, it accounts for upwards of 42% of combat-related deaths [Shear, 2013 #3392]. Blast-induced mild TBI has emerged as the 'signature injury' from Iraq and Afghanistan [Levin, 2013 #3184]. TBI can be classified as primary, resulting from the initial trauma, or as secondary, relating to intracranial pressure elevations, hemorrhage and edema, cortical spreading depolarization waves, temperature dysregulation, ischemia, hypoxia, cellular ionic and metabolic derangements, neuroinflammation, coagulopathy, neurogenic organ dysfunction and/or hypotension occurring as consequences of the primary insult. TBI also predicts the development of both post-traumatic stress disorder and attention deficit hyperactivity disorder.



The above diagram illustrates the time to death from admission from a retrospective review of 1,029 deaths over 4 years at a single large urban trauma center—the University of Texas Houston, Texas [Pati, 2015 #4373]. The majority of civilian deaths occur very early, 40% after day 1, and 26% after day 3. In contrast, on the battlefield, the vast majority of deaths occur within the Golden Hour before reaching a MTF (in black) illustrating the major differences in operational windows-of-care compared to the civilian setting.

Controversy over Hextend® use in Prehospital Military Medicine: In 2010 amidst the growing clinical concerns with hydroxyethyl starch (HES) solutions [Lissauer, 2011 #2799;Haut, 2011 #2902;Mutter, 2013 #4062], the TCCC continued to endorse the use of 500 ml (max 1L) Hextend® for the Special Forces in austere environments. In June 2013 the FDA "black boxed" 6% HES with the warning that it should not be used to treat patients with hypovolemia or the critically ill (e.g., those with sepsis) or patients undergoing cardiac surgery [Dobson, 2014 #3143]. In the same month, the European Medicines Agency formally suggested that the colloid be banned altogether. The current TCCC guidelines have relegated Hextend® as a third option when whole blood or blood products are not available [Butler, 2014 #4057;Cap, 2015 #4139].

Purpose of our Study: To develop a small-volume 'one-two' fluid therapy using 3% NaCl adenosine, lidocaine and Mg^{2+} (ALM) bolus and 0.9% ALM drip for: 1) resuscitation after severe uncontrolled blood loss in the rat model of liver resection with or without TBI and, 2) a *continuum-of-care* stabilization IV 'drip' to reduce secondary-hit complications. The effects of ALM on survival, bleeding, hemodynamics, inflammation and organ injury were compared with Hextend and Saline controls in our model. Beta-hydroxybutyrate was added to the ALM in the TBI study to test for additional benefits.

Scope: To reduce preventable mortality and morbidity in combatants suffering severe hemorrhagic shock with or without suspected TBI at the point-of-injury through to definitive care.

2. KEYWORDS:

Small-volume, resuscitation, hypotensive, non-compressible, truncal, hemorrhage, TBI, coagulopathy, inflammation, ALM, adenosine, lidocaine, magnesium, ROTEM, cardiac, neurogenic, multiple-organ failure, Hextend, fluid therapy

3. ACCOMPLISHMENTS:

a. **What were the major goals of the project *as stated in the approved SOW*?**

Specific Goal # 1: To demonstrate that 0.7 ml/kg 3% NaCl ALM fluid can raise mean arterial blood pressure (MAP) into the hypotensive range and protect heart and brain following uncontrolled blood loss and TBI in the rat model. We will also test the effect of ALM with beta-hydroxybutyrate, an important fuel for the brain and possible neuroprotectant.

Specific Goal # 2: To demonstrate the efficacy of a "one-two" strategy in the rat model with the bolus being followed by an ALM drip for longer-term whole body stabilization times urgently required in far forward settings with delayed evacuation times.

Specific Goal # 3: In addition to providing cardiac and hemodynamic support to brain and renal function during uncontrolled blood loss and TBI, our third goal was to demonstrate ALM's front-line anti-ischemic, anti-inflammatory, and coagulation restorative properties to reduce secondary complications after uncontrolled blood loss with and without TBI.

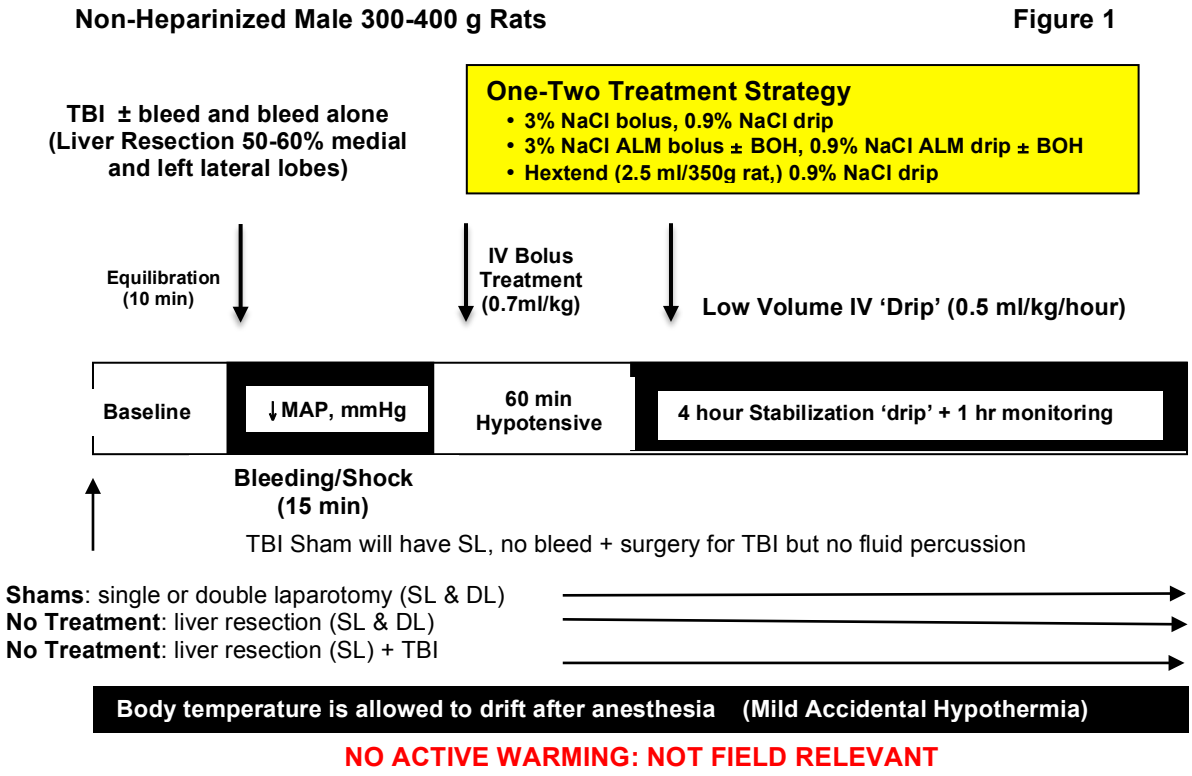
b. **What was accomplished under these goals?**

1) Major activities; All three specific goals/aims were met.

2) Specific objectives; All specific objectives were met.

Methodology: Rat model of uncontrolled blood loss, shock with and without TBI (Fig 1).

- **Uncontrolled Hemorrhage and Shock:** was produced by severing 50-60% of median and left lateral liver lobes (30-40 ml blood loss/kg). No lost blood was returned.
- **Traumatic Brain Injury:** TBI was induced using the lateral fluid percussion model receiving mild to moderate trauma induced with force of 2.3 to 2.5 atm (Dragonfly HPD-1700 Fluid Percussion Device).



c. **Major Findings, Developments, and Conclusions**

This section of the report will be under 3 major headings:

- **STUDY 1:** 'Non-Compressible' Hemorrhagic Shock
- **STUDY 2:** Traumatic Brain Injury
- **STUDY 3:** Combined Traumatic Brain Injury + Hemorrhagic Shock

Executive Summary

The 12-month USSOCOM funded study addressed three military capability gaps relating to severe shock, control of internal hemorrhage and TBI, singly or in combination. We showed that small-volume 3% NaCl ALM bolus (0.7 ml/kg IV) and 0.9% NaCl ALM 'drip' (0.5 ml/kg/hour for 3 to 4 hours): 1) resuscitated MAP and reduced truncal hemorrhage by up to 60% after liver resection and shock, 2) reduced secondary injury and improved cardiac function after mild-to-moderate traumatic brain injury (TBI), and 3) led to a 50% reduction in mortality, 50% reduction in internal blood loss compared to 3% NaCl controls after combined TBI and hepatic hemorrhage. Hextend resuscitation, administered as a bolus according to TCCC guidelines, promoted internal bleeding, inflammation, organ failure and early death. The multi-faceted frontline ALM strategy appears to arise from its ability to improve cardiac and hemodynamic function, correct coagulopathy, improve platelet function, reduce inflammation and improve tissue oxygenation. Small-volume ALM resuscitation therapy may provide SOF combat medic/corpsman with a new "One-Solution-Fits-All" to improve warfighter survivability in far-forward environments.

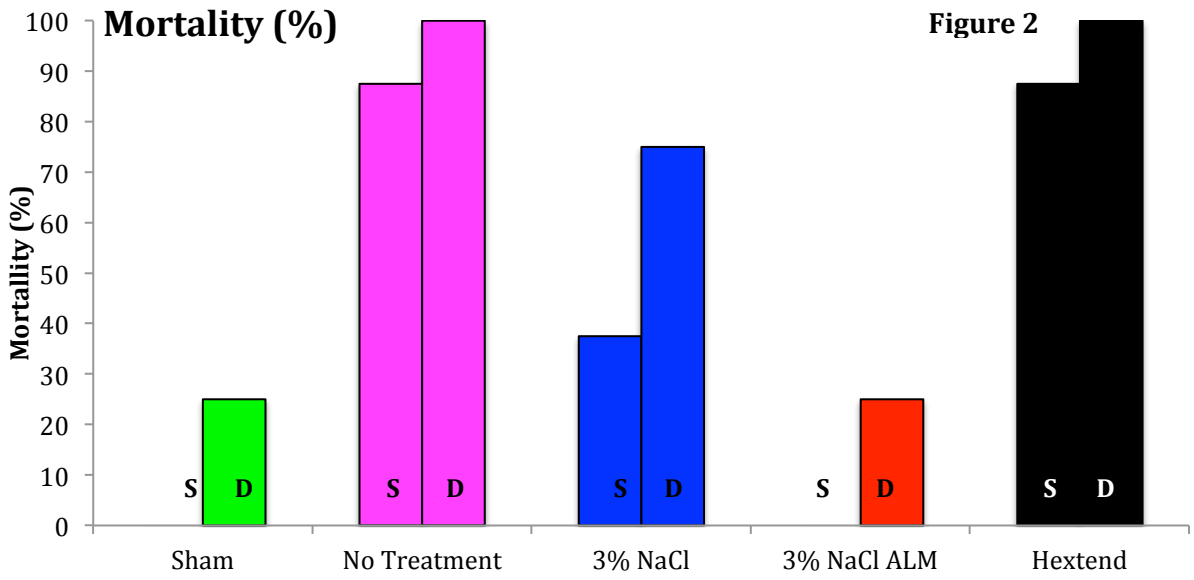
STUDY 1

'Non-Compressible' Hemorrhagic Shock

Additional data for the major findings in STUDY 1 are found in Appendices 1-13 (p41-53).

1. Survival Outcomes

ALM bolus-infusion treatment significantly improved survival compared to Untreated animals, 3% NaCl Controls or Hextend treatment at TCCC recommendations.



S = single laparotomy; **D** = double laparotomy; **Sham** = Surgery No Bleed. **No Treatment**: Surgery + liver bleed

NB: The placement of kidney and gut flow probes/O_2 sensor required a second laparotomy (paramedian incision) in n=8 animals for each group (Sham, No Treatment, 3% NaCl, 3% NaCl ALM, and Hextend). Data are presented for single laparotomy (S) and double laparotomy (D). **Sham** = Surgery No Bleed. **No Treatment**: Surgery + liver bleed. Sham animals received identical treatment to 3% NaCl controls (0.7 ml/kg 3% NaCl bolus Phase 1, 0.5 ml/kg/hr 0.9% NaCl drip Phase 2). No Treatment animals underwent surgery + liver resection and uncontrolled bleeding *without fluid resuscitation* and were monitored for 60 min Phase 1 and 300 min Phase 2.

Mean Survival Time (min from liver injury)		
Group	Single Laparotomy (SL)	Double Laparotomy (DL)
Sham	375±0	357±14
No Treatment	117±33*	149±34*
3% NaCl	286±49	253±45
3% NaCl ALM	375±0	354±14
Hextend	82±42[#]	78±18[†]

* $p<0.05$ compared to Sham and 3% NaCl ALM
[#] $p<0.05$ compared to all other groups
[†] $p<0.05$ compared to Sham, 3% NaCl, and 3% NaCl ALM

Note: Maximum possible time for survival was 375 min and protocol driven after liver resection (See Fig 1, p5)

- **Mortality Sham** (no liver resection) resulted in 0% and 25% mortality single laparotomy (SL) and double laparotomy (DL) respectively (Fig 2, p7). NOTE: Some blood loss occurred in Sham animals undergoing double laparotomy as a result of trauma of the paramedian incision required for placement of kidney and gut flow/pO_2 probes.

- **Mortality No Treatment** produced 87.5% and 100% mortality for SL + liver resection and DL + liver resection respectively (Fig 2, p7).

- **Mortality Saline Controls** (3% NaCl bolus and 0.9% NaCl 'drip') led to 38% and 75% mortality for SL and DL respectively. Mean survival times of 286 and 253 min single and double laparotomy respectively, were significantly longer than Hextend group.

- **Mortality ALM treatment** (3% NaCl ALM bolus and 0.9% NaCl ALM 'drip') led to 0% and 25% mortality for SL and DL groups (Fig 2, p7). ALM mortality was the same as Sham. Mean survival time in ALM group was significantly longer than Untreated animals and Hextend group.

Kaplan-Meier survival plots for single laparotomy (Fig 3) and double laparotomy (Fig 4) groups are shown over (p8).

Major Finding:

Hextend-treated rats died EARLY after 60 min hypotensive resuscitation (Phase 1) and had a worse outcome than NO TREATMENT in terms of earlier deaths (same % mortalities).

- Mortality from Hextend (78-82 min after liver injury) was worse than NO TREATMENT (No Treatment deaths occurred at 117 and 149 min after liver resection).

- These results provide further support to the current literature showing deleterious effects associated with the use of Hextend.

Note: Maximum possible time for survival was 375 min and set by the protocol after liver resection (See Fig 1, p5).

Figure 3

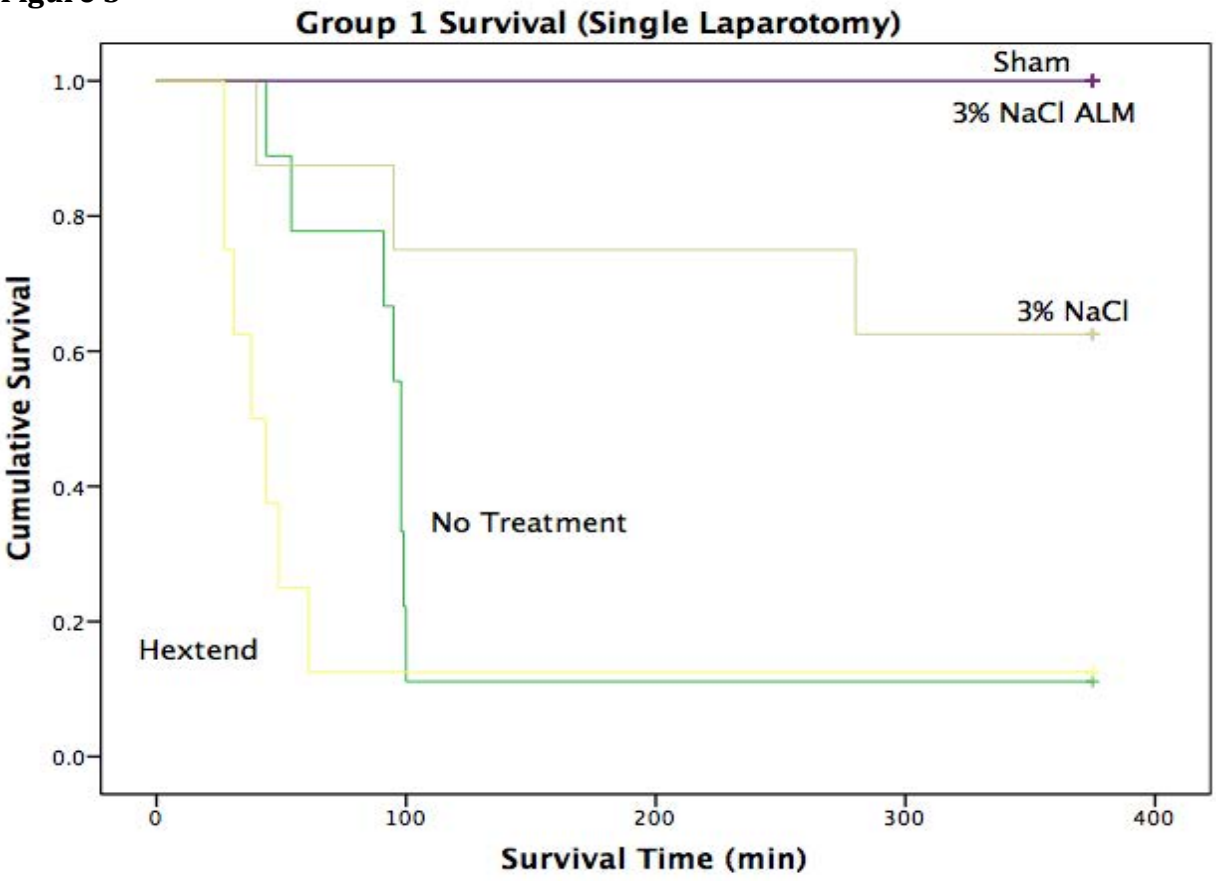
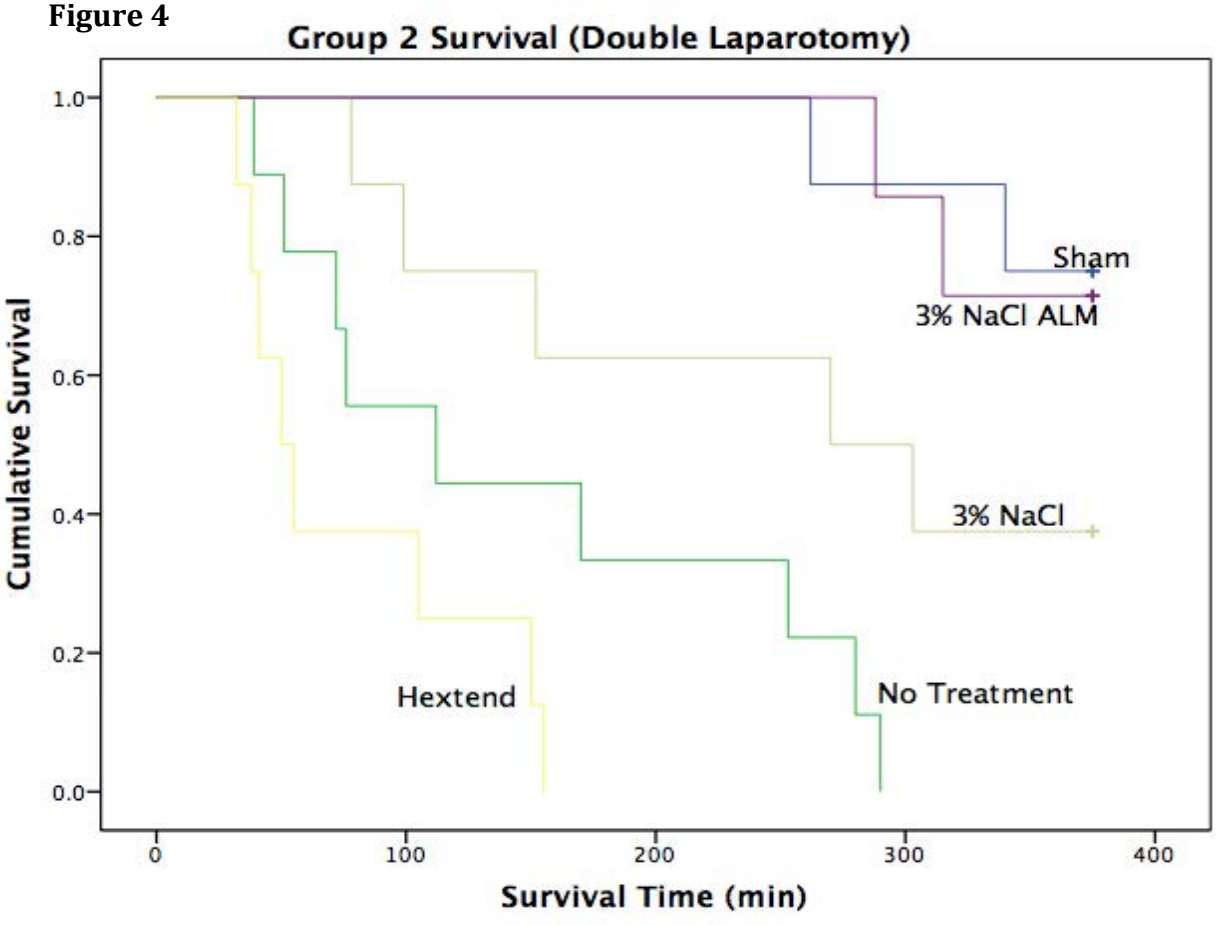


Figure 4



2. Non-Compressible Blood Loss

Blood loss, expressed as a percentage of total blood volume, was estimated at time of sacrifice by mopping up clots and loose blood in the abdominal cavity with pre-weighed gauze. Total blood volume for the rat was estimated from the relationship 0.06 x body weight + 0.77.

Major Finding:

- **3% NaCl ALM treatment led to 40% less blood loss compared to SALINE controls (single laparotomy groups)**
- **3% NaCl ALM treatment led to 60% less blood loss compared to SALINE controls (double laparotomy groups) or NO TREATMENT (liver resection without fluid resuscitation).**

- 3% NaCl ALM bolus-infusion treatment resulted in 40 to 60% less blood loss (375 min after liver resection) compared to 3% NaCl controls. 3% NaCl controls had 15% and 27% blood loss, and ALM-treated rats had 9% and 11% after single and double laparotomy, respectively. ALM led to 9/15% = 60% and 11/27% (40%) bleeding compared to 3% NaCl.

- SHAMS (surgery but no liver resection) with 3% NaCl bolus and 0.9% NaCl infusion had 3% blood loss from a single laparotomy, and 15% blood loss after the second laparotomy. The experiment shows that the second laparotomy (3 cm paramedian incision) and tissue trauma required for flow/pO_2 probe insertion into kidney and mesentery resulted in 5-times more bleeding than single transverse laparotomy.

- No treatment (liver resection/no treatment) had 19% and 31% blood loss after single and double laparotomy.

Interpretation: 3% NaCl ALM treatment appears to act like a "pharmacological tourniquet" for non-compressible bleeding. This may be due to the drug's ability to rapidly correct coagulopathy and blunt systemic inflammation. ALM-treated animals had up to 60% less blood loss than 3% NaCl controls. A second laparotomy which caused 5-times more blood loss and trauma in SHAMS did not significantly increase blood loss in ALM rats compared to the first laparotomy after liver resection. This is another potentially important ALM finding for combat medics/corpsmen and field surgeons in the early treatment of combatants suspected for severe internal bleeding at the point-of-injury.

Major Finding:

Hextend-treated animals had 23% blood loss after the single laparotomy and 44% blood loss after double laparotomy. No treatment (liver resection without fluid resuscitation) had 19% and 31% blood loss respectively. ALM blood loss was 9% and 11% at 375 min after liver resection. Hextend-treated animals also died earlier than any other group i.e. ~ 80 min after liver resection with a bolus administration recommended in the current TCCC guidelines (single bolus 500 ml/70 kg human or 7 ml/kg). We concluded that early death in Hextend-treated animals was primarily from the failure of Hextend to resuscitate the heart (cardiovascular collapse), and from severe derangements to the coagulation and inflammatory systems.

Figure 5: Representative Examples of Blood Loss

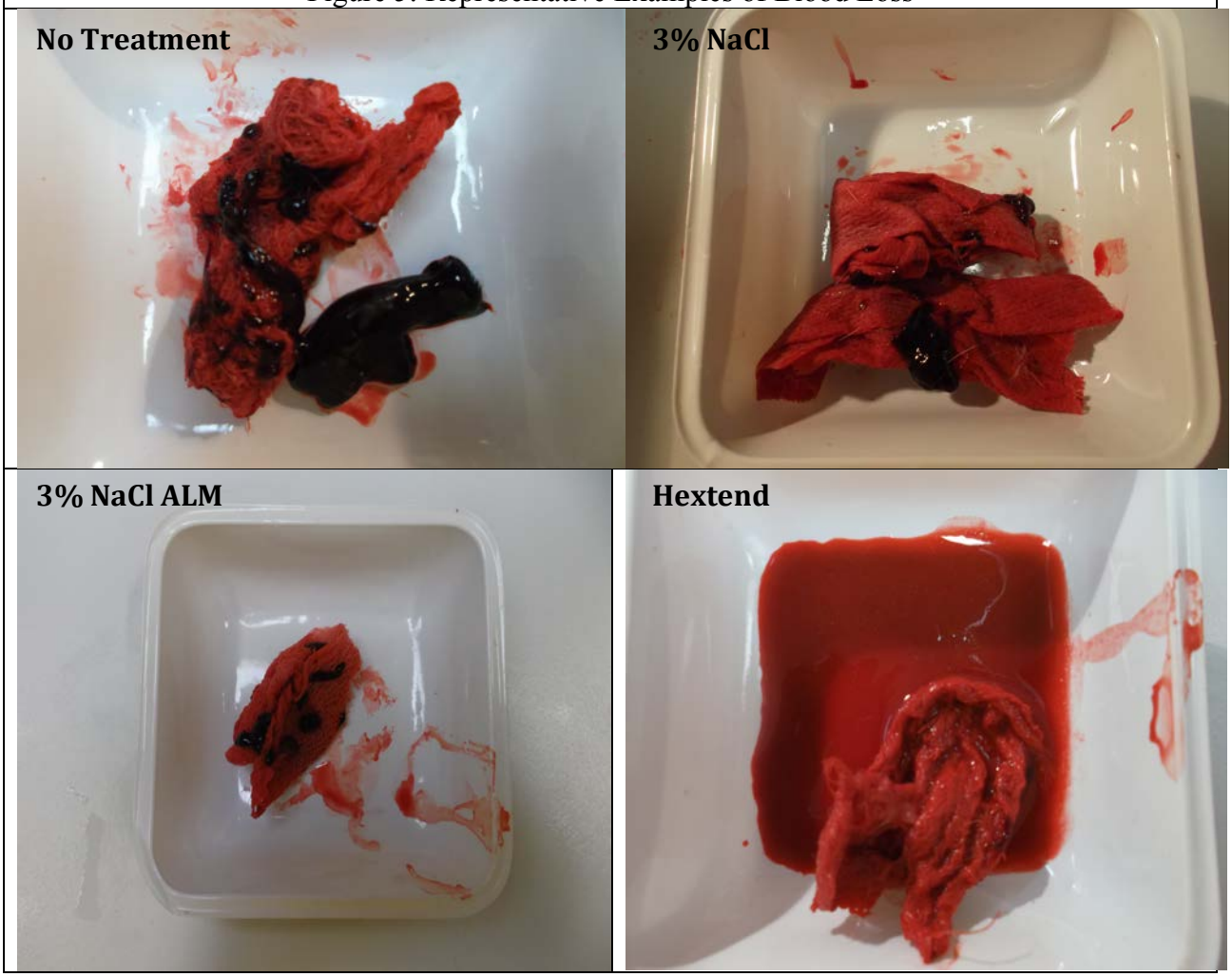


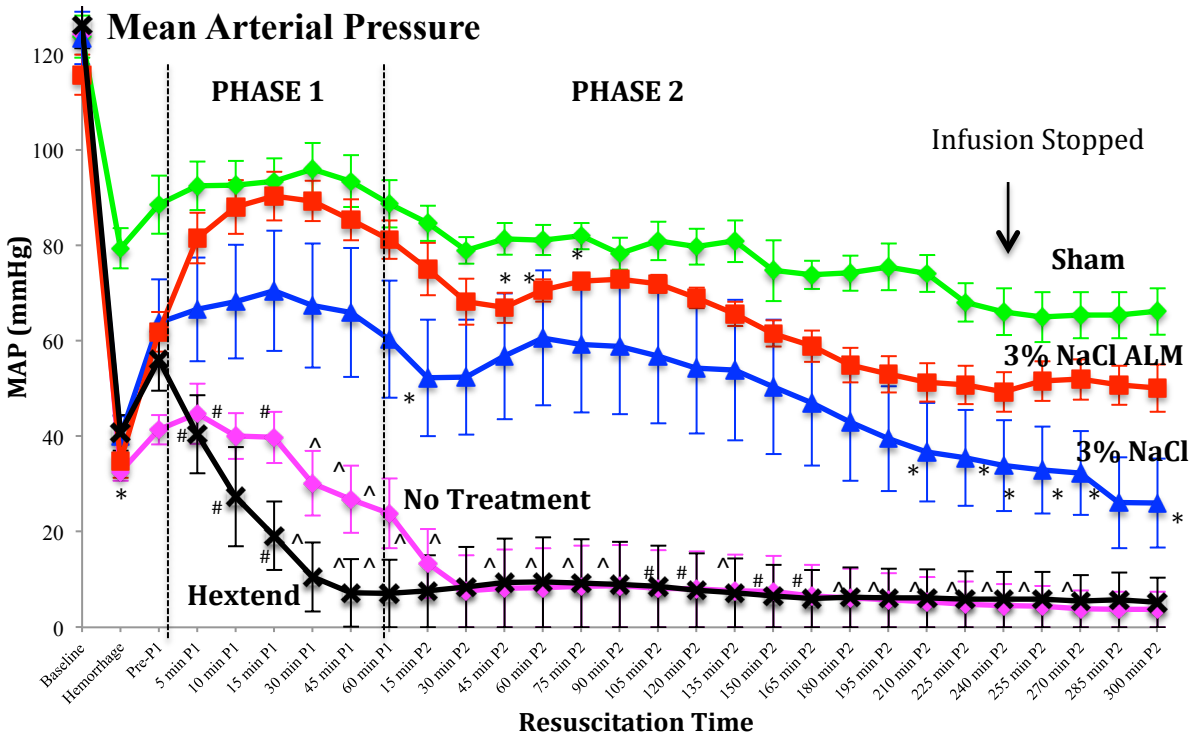
Figure 5 show photos of the blood mopped up using pre-weighed gauzes from the abdominal cavity at the time of death or termination of the experiment as per protocol. The photos clearly show the hypocoagulable properties of Hextend-treated animals. ALM-treated animals had significantly less blood compared to Hextend or controls.

3. Hemodynamic and Metabolic Outcomes during Phase 1 Resuscitation

Major Finding:

ALM bolus-infusion treatment led to more stable MAP, defended body temperature, and improved acid-base status with higher pH, lower blood lactate levels and improved electrolytes (e.g. lower plasma K^+).

i) Mean Arterial Pressure



* $p<0.05$ c.f. Sham
# $p<0.05$ c.f. Sham and 3% NaCl ALM
^ $p<0.05$ c.f. Sham, 3% NaCl, and 3% NaCl ALM

MAP (mmHg)	Sham	No Treatment	3% NaCl	3% NaCl ALM	Hextend
60 min Phase 1	89±5	24±7^	60±12	81±4	7±7^
300 min Phase 2	66±5	4 (n=1)	26±9*	50±5	5 (n=1)

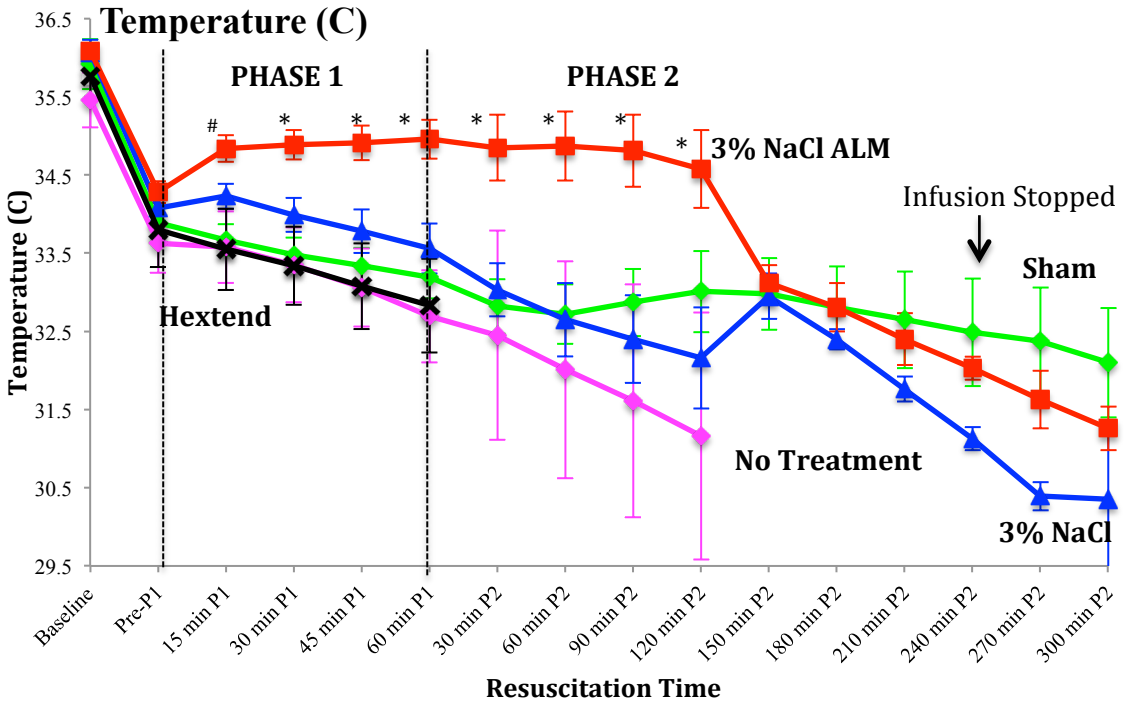
Data represent mean±SEM mean arterial pressure (MAP, mmHg) in animals undergoing single laparotomy (Sham) or single laparotomy with liver resection and uncontrolled bleed (No Treatment, 3% NaCl, 3% NaCl ALM, Hextend).

- 0.7 ml/kg 3% NaCl ALM bolus resuscitated MAP after uncontrolled hepatic hemorrhage (MAP<40 mmHg) over 60 min Phase 1.

- After 60 min Phase 1, 0.5 ml/kg/hr 0.9% NaCl ALM "drip" maintained MAP not significantly different from Shams throughout Phase 2 resuscitation, whereas in Saline controls 0.9% NaCl drip could not sustain MAP which steadily decreased toward shock levels.

ii) Body Temperature

Animals were not actively warmed at any time during surgery or the experimental period in order to mimic the field setting.



* $p<0.05$ compared to all other groups
\# $p<0.05$ compared to Sham and No Treatment group

- 3% NaCl ALM bolus maintained a significantly higher body temperature compared to all other groups, including Shams, throughout 60 min Phase 1 resuscitation.
- 0.9% NaCl ALM infusion maintained a significantly higher body temperature compared to all other groups for the first two hours of Phase 2 resuscitation.

By maintaining a higher body temperature (mild hypothermia ~35°C) ALM appears to be acting like a "*pharmacological thermoregulator*" during hemorrhage and shock, which may be beneficial to the bleeding combatant. It is interesting that in ALM-treated animals hypothermia was not associated with metabolic acidosis or coagulation disturbances (See below and Section 5: Coagulopathy, p16-17).

iii) Acid-Base Balance and Lactate

Hydrogen ion (pH), Lactate, Base Excess and bicarbonate values at baseline, after 60 min Phase 1 resuscitation, and every 60 min throughout Phase 2 resuscitation are shown in Appendix 1 (p41).

- Blood lactate levels in 3% NaCl ALM treated animals were lower than controls after liver resection and uncontrolled hemorrhage. After 60 min Phase 1, lactate rose only 9% from baseline (2.84±0.28 vs. 2.61±0.14 mM) in contrast to 3% NaCl controls (146%↑ from 2.11±0.31 to 5.20±1.34), Untreated animals (45%↑ from 2.10±0.23 to 3.05±0.75), and Hextend-treated animals (273%↑ from 1.83±0.18 to 6.83±1.74, $p<0.05$ c.f. 3% NaCl ALM).

iii) Electrolytes

Plasma K^+, Na^+, Ca^{2+}, and Cl^- levels at baseline, after 60 min Phase 1 resuscitation, and every 60 min throughout Phase 2 resuscitation are shown in Appendix 2 (p42).

- In contrast to all other groups, including Sham, 3% NaCl ALM bolus resulted in a decrease in plasma K^+ (4.27±0.15 to 4.01±0.12 mM).
- ALM drip maintained *lower K^+ levels* compared to all other groups.

Interpretation: A lower plasma K^+ may reflect less whole body tissue ischemia during ALM treatment because ischemic cells are known to release K^+ into plasma as they depolarise their cell membrane potentials. That ALM defends plasma K^+ from rising in blood is also consistent with lower lactate levels from improved supply and demand relationship from higher pO2 and blood flows, and lower demand in kidney and gut (p21-22), and near normal blood pH (pH 7.3) compared to controls which were acidotic (pH 7.1) (Appendix 1, p41)

4. Cardiac Function

Major Findings:

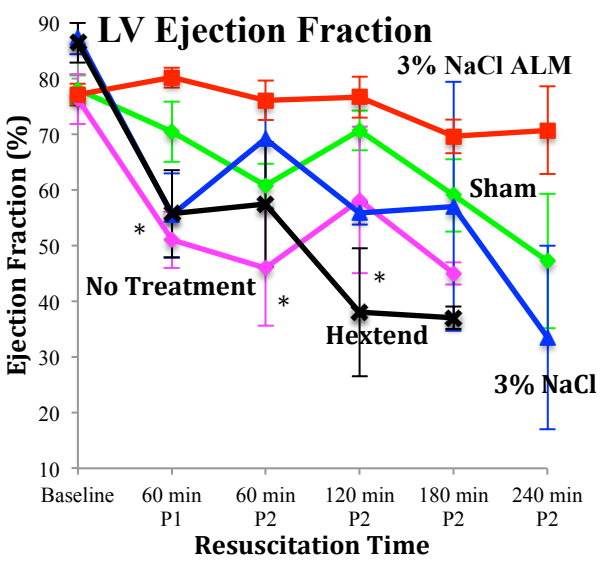
ALM-treated animals had improved cardiac function with significantly increased cardiac output, stroke volume, cardiac contractility and ejection fraction.

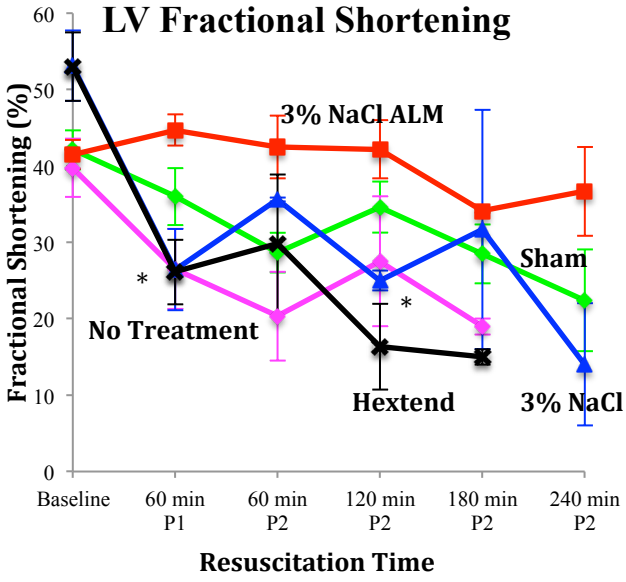
ALM bolus-infusion therapy maintained a stable heart rate with significantly fewer arrhythmias compared to 3% NaCl controls, Not Treated animals and Hextend-treated animals.

Transthoracic echocardiographic imaging and calculations were performed according to the guidelines of the American Society of Echocardiography using a 13 MHz linear array transducer (SL1543) and MyLab Gold ultrasound (Esaote, Genova, Italy). Two-dimensional parasternal long- and short-axis views and two-dimensional targeted M-mode tracings were taken at baseline, 60 min Phase 1 Resuscitation, and every 60 min during Phase 2 Resuscitation. Parameters measured include percentage of left ventricular (LV) fractional shortening (FS, %), ejection fraction (EF, %), internal dimensions of the left ventricle at both diastole and systole (LVIDd and LVIDs, mm), posterior wall dimensions at diastole and systole (LVPWd and LVPWs, mm), and diastolic and systolic interventricular septal dimensions (IVSd and IVSs, mm). Diastolic (EDV) and systolic (ESV) volumes (ml) are calculated from the equations $1.047[LVIDd]^3$ and $1.047[LVIDs]^3$, respectively. Cardiac output (CO, ml/min) is calculated as stroke volume (SV) x heart rate (HR), where SV = EDV – ESV.

Group	Time	Heart Rate (bpm)	Stroke Volume (ml)	Cardiac Output (ml/min)
Baseline	Sham	339±12	0.19±0.04	64.4±12.5
	No Treatment	334±27	0.16±0.02	57.1±5.8
	3% NaCl	333±10	0.13±0.05	42.2±15.2
	3% NaCl ALM	344±7	0.16±0.02	54.4±8.5
	Hextend	326±9	0.15±0.06	48.1±19.2
60 min Phase 1	Sham	287±16	0.15±0.02	43.3±7.4
	No Treatment	258±23	0.07±0.01*	17.7±4.1*
	3% NaCl	269±18	0.09±0.03	25.2±7.2
	3% NaCl ALM	295±7	0.21±0.04	60.9±10.3
	Hextend	252±39	0.04±0.01#	9.93±3.2#
60 min Phase 2	Sham	282±16	0.11±0.02	31.1±4.3
	No Treatment	287±7	0.05±0.02	14.9±5.8
	3% NaCl	275±17	0.07±0.03	20.7±8.0
	3% NaCl ALM	288±9	0.18±0.04	49.6±10.2
	Hextend	286±8	0.04±0.01	10.9±3.7*
120 min Phase 2	Sham	265±18	0.19±0.05	47.4±12.0
	No Treatment (n=3)	261±11	0.11±0.09	27.6±23.0
	3% NaCl	246±14	0.13±0.07	34.0±17.8
	3% NaCl ALM	264±12	0.16±0.04	41.1±10.1
	Hextend (n=2)	250±10	0.05±0.04	12.2±9.0
180 min Phase 2	Sham	264±19	0.20±0.05	55.0±17.2
	No Treatment (n=2)	242±17	0.15±0.09	34.2±20.3
	3% NaCl	218±28	0.07±0.04	16.4±10.7
	3% NaCl ALM	265±25	0.15±0.06	44.8±19.0
	Hextend (n=2)	172±17	0.065±0.03	10.4±5.5

* $p<0.05$ c.f. 3% NaCl ALM
# $p<0.05$ c.f. Sham and 3% NaCl ALM





* $p<0.05$ c.f. 3% NaCl ALM

5. Coagulopathy

Major Finding 1:

ALM treatment fully corrected PT, aPTT and ROTEM-assessed coagulopathy (Phase 1 and 2) compared to 3% saline controls.

- **Controls showed a worsening hypocoagulopathy.**
- **ALM correction was associated with 2 to 3x higher plasma fibrinogen levels compared to 3% NaCl controls and Hextend group.**

Major Finding 2:

Hextend resuscitation administered according to TCCC guidelines was profoundly hypocoagulable, which was associated with early death. Hextend-treated animals also had significantly lower plasma fibrinogen levels compared to Sham, Controls and ALM treatment.

Phase 1 and Phase 2 ROTEM (EXTEM, INTEM, FIBTEM, and APTEM tests) and Stago (PT, aPTT, and Fibrinogen) data can be found in Appendices 3-7 (p43-47). Representative combined TEMograms for each group after Phase 1 and Phase 2 resuscitation are shown over (p17).

Plasma Fibrinogen levels were significantly higher during 3% NaCl ALM resuscitation

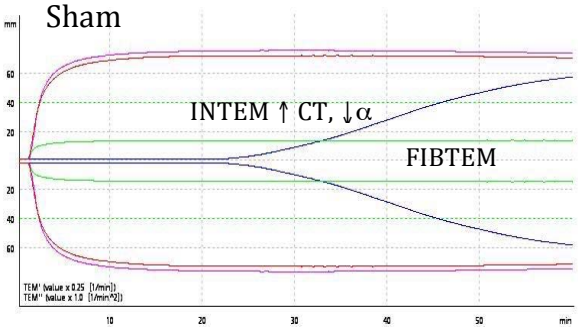
Group	PT (sec)		aPTT sec)		Fibrinogen (g/dL)	
	Phase 1	Phase 2	Phase 1	Phase 2	Phase 1	Phase 2
Baseline	15±0.3		35±2		2.43±0.05	
Sham	41±15	94±36	144±56*	130±37	1.37±0.33	1.43±0.40
No Treatment	43±22*	23±5 (n=3)	79±27*	64±7 (n=3)	1.05±0.39#	1.86±0.46 (n=3)
3% NaCl	30±3	165±15 (n=5)	91±38	177±23† (n=5)	0.97±0.21#	0.45±0.05 (n=5)
3%NaCl ALM	17±0.2	41±7	39±3	100±23	1.87±0.11	1.39±0.11
Hextend	56±17‡	102±73 (n=2)	132±24*	191±9 (n=2)	0.64±0.09¶	0.75±0.34 (n=2)

* $p<0.05$ compared to Baseline and ALM
# $p<0.05$ compared to Baseline
† $p<0.05$ compared to ALM
¶ $p<0.05$ compared to Baseline, Sham and ALM
‡ $p<0.05$ compared to Baseline, Sham, Control, and ALM

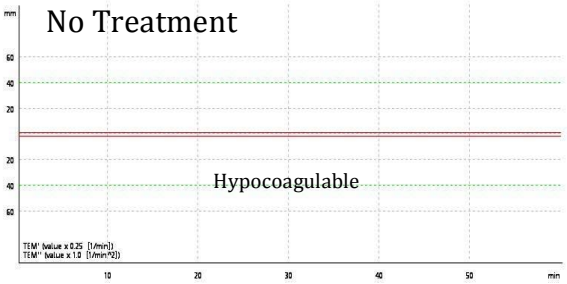
Representative TEMograms

PHASE 1 RESUSCITATION

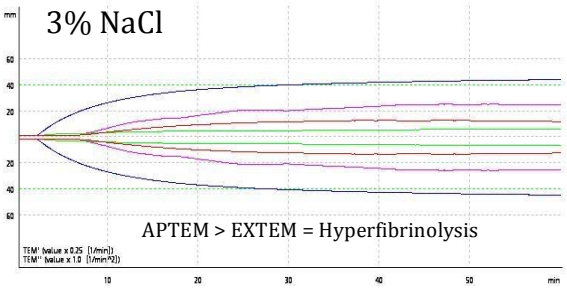
Sham



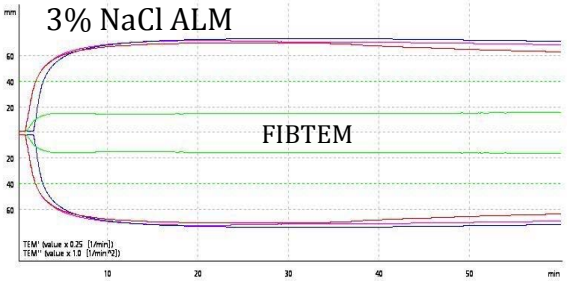
No Treatment



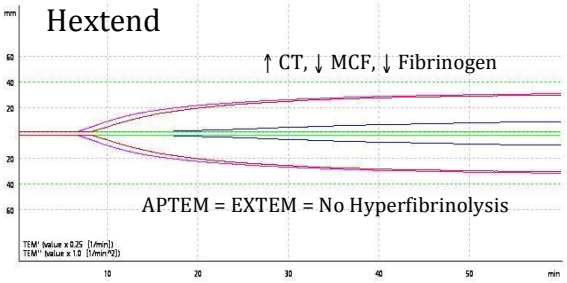
3% NaCl



3% NaCl ALM

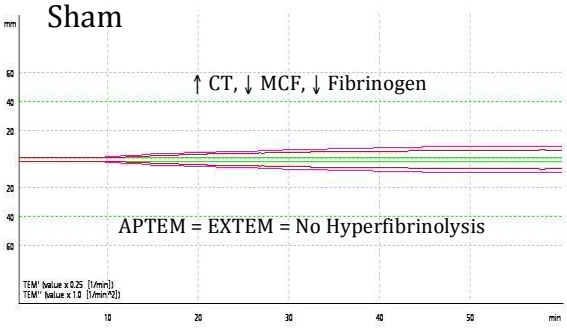


Hextend

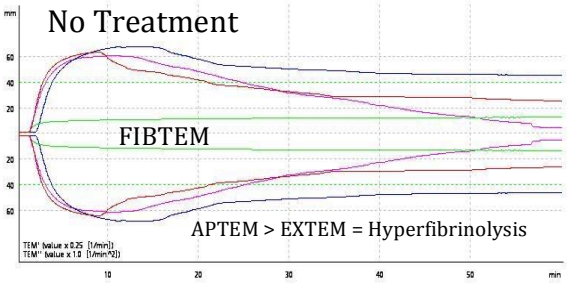


PHASE 2 RESUSCITATION

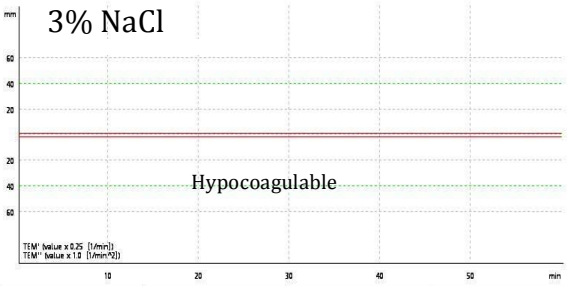
Sham



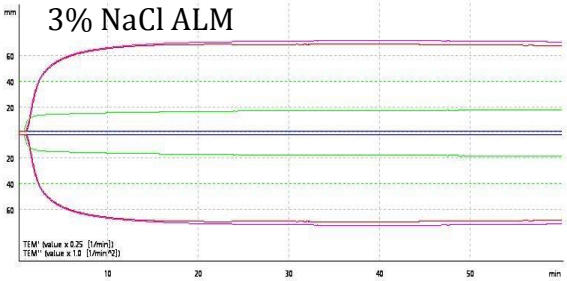
No Treatment



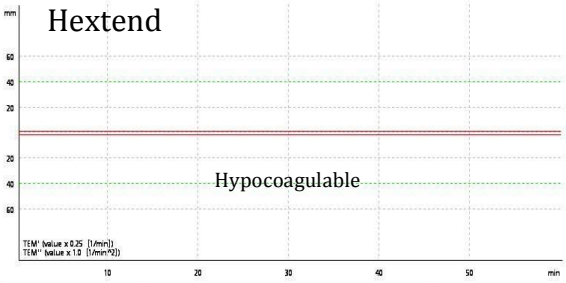
3% NaCl



3% NaCl ALM



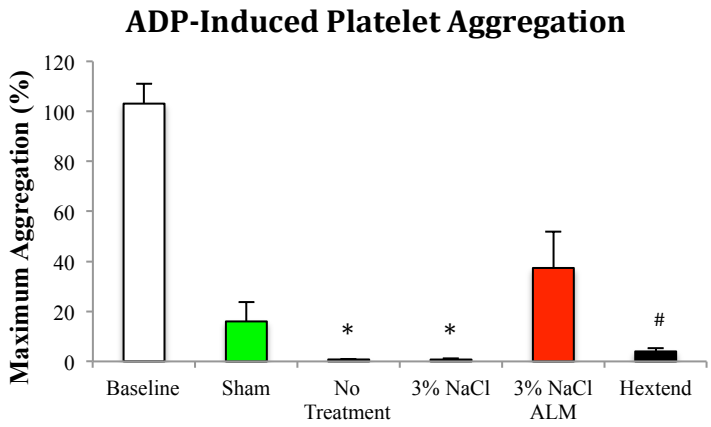
Hextend



6. Platelet Function:

Major Finding:

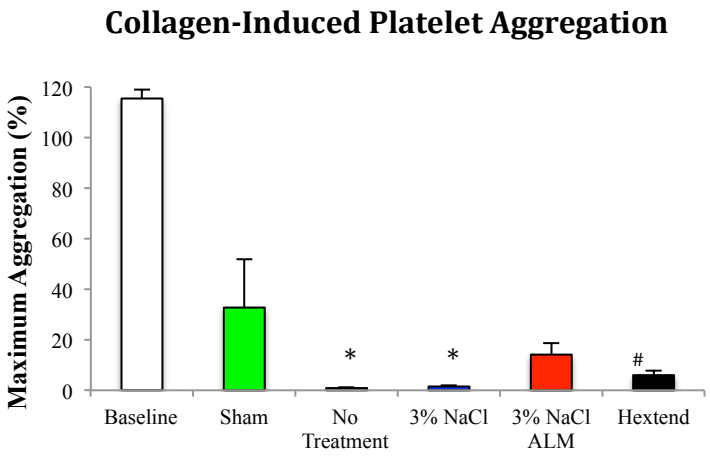
Uncontrolled hemorrhage severely compromised platelet aggregation. ALM treatment significantly preserved ADP- and collagen-activated platelet function compared to Untreated animals, 3% NaCl controls, and Hextend-treated animals.



* $p<0.05$ c.f. Sham and 3% NaCl ALM groups

$^{\#}$ $p<0.05$ c.f. 3% NaCl ALM

All groups significantly reduced compared to Baseline



* $p<0.05$ c.f. Sham and 3% NaCl ALM groups

$^{\#}$ $p<0.05$ c.f. 3% NaCl ALM

All groups significantly reduced compared to Baseline

Additional data including primary slope and disaggregation shown in Appendix 8 (p48).

7. Inflammation and Organ Injury

i) Systemic Inflammation

Major Finding:

ALM-treated animals had significantly lower systemic pro-inflammatory cytokines/chemokines and interferons (IL-1 alpha and beta, TnF-alpha, IL-6 and RANTES, IFN-gamma) compared to 3% saline controls or the Hextend group. Saline controls and Hextend fluid therapy led to a pro-inflammatory state, which worsened during Phase 2 resuscitation.

Our data indicated that 3% NaCl ALM bolus-infusion therapy blunted the early activation of key pro-inflammatory cytokines, chemokines and interferons and their respective receptor networks presumably from improved cardiac function and invoking less secondary injury during uncontrolled bleeding and hemorrhagic shock. Both 3% Saline controls and Hextend treatment showed increased inflammation and injury profiles (↓ survival, ↓ cardiac resuscitatability, ↑ coagulopathy, ↑ liver damage (AST, ALT), ↑ renal damage (NAG), ↑ blood lactate, ↑ plasma K^+ and ↓ blood pH) compared to ALM-treated animals. It is also of interest that inhibition of cardiac contractility (prevention of myocardial depression) and hemodynamic compromise has been associated with increases in cytokines TNF-α, IL-1β and IL-6. These cytokines are also known to increase the hypothalamic–pituitary–adrenal (HPA) axis stress hormone production and increased sympathetic to parasympathetic flows, which can exacerbate inflammation and multiple organ dysfunction.

Summary Table: Cytokines, Chemokines and Interferons in Phase 1 and 2 Resuscitation
(for Details see Appendix 9, p49)

(pg/ml)	**60 min Phase 1 (bolus)**			**300 min Phase 2 (drip)** (or death)		
	3%NaCl Control (6)	**3%NaCl ALM (8)**	**Hextend**	**3%NaCl Control (6)**	**3%NaCl ALM (8)**	**Hextend**
Cytokine						
IL-1 alpha	205±132	ND (<3.3)	297	445±84	22±19 (5%) (ND; n=4)	885
IL-1 beta	50±38	ND (<1.3)	166	618±85	32±22 (5%)| (ND; n=5)	830
TNF-alpha	46±11	11±4 (24%) (ND; n=1)	15	33±6	4±1 (12%)	38
Chemokine						
RANTES	869±308	298±86 (34%) (ND; n=1)	1099	1818±312	637±140 (35%) (ND n=3)	3318
IL-6	1324±871	82±7 (6%) (ND; n=3)	633	110,931±21,244	9142±4000 (8%)	172,100
Interferon						
IFN-gamma	18±11	3±2 (17%) (ND; n=4)	22	208±100	11±3 (5%) (ND; n=2)	255

ND: Not Detected
ALM group (% control in parentheses)
ND and n in parentheses refers to number of animals where the cytokine, chemokine or interferon levels were not detected

IL-1 alpha and beta, IL-6 and TNF-alpha are "trigger" cytokines that play a pivotal role in the activation of the inflammatory response to injury. IL-6 has pro- and anti-inflammatory properties and these cytokines are now regarded as prominent targets for clinical intervention. IL-6 also stimulates the production of acute phase proteins, and induces leukocytosis, fever, and angiogenesis. Also known to trigger the HPA-axis and stress response to trauma.

RANTES (Regulated on Activation, Normal T Cell Expressed and Secreted), also known as CCL5, is a chemokine and mediator of acute inflammation at specific tissue sites.

IFN-gamma (originally called macrophage-activating factor) is an interferon that coordinates a diverse array of cellular programs through transcriptional regulation of immunologically relevant genes through the Jak-Stat pathway. It helps orchestrate the trafficking of specific immune cells such as unstimulated leukocytes to sites of injury/inflammation through up-regulating expression of adhesion molecules and chemokines. Unstimulated leukocytes cycle continuously between the blood and the lymph. IFN-γ regulates this process by up-regulating expression of chemokines (e.g., IP-10, MCP-1, MIG, MIP-1α/β, RANTES) and adhesion molecules (e.g., ICAM-1, VCAM-1). TNF-α and IL-1β also *synergistically* regulate many of these molecules.

Interpretation: The early attenuation of the pro-inflammatory response to trauma with ALM treatment may contribute to improved cardiac and multiple organ protection and improved survival. ALM may also reduce the trauma-induced activation of the HPA axis which also blunts the inflammatory response.

ii) Liver Damage

Major Finding:

3% NaCl ALM had significantly less damage than controls based on liver injury markers aspartate and alanine aminotransferases.

Increased liver injury markers Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) in saline controls indicate acute liver injury (Appendix 10 (p49).). AST is released into the bloodstream proportional to liver cellular damage and cellular necrosis or destruction of hepatocytes, and ALT is a marker of liver inflammation. Kidney levels of N-acetyl-beta-D-glucosaminidase (NAG) also are a marker of injury. Hextend treatment led to increased liver injury markers and significantly higher plasma NAG levels. Increased NAG levels support current knowledge of a nephrotoxic effect associated with Hextend use, and may also indicate worsened pulmonary and cardiac damage and contribute to early mortality.

iii) Oxidative Stress and Pulmonary Edema

Hextend, administered also resulted in increased oxidative stress and pulmonary oedema associated with early mortality. ALM therapy attenuated oxidative stress compared to Untreated animals, 3% NaCl controls and Hextend-treated animals (Appendix 11, p50)

8. Tissue pO_2 and Blood Perfusion from Laser Doppler Flowmetry

Background: Tissue pO_2 and blood perfusion were measured in gut, kidney, and muscle with the Oxford Optronix pO_2/Flow 'Bare-Fibre' sensor connected to Oxylite Pro XL ad Oxyflo Pro XL for data recording. Gut tPO_2 and flow was measured at the same location in each animal in the small intestine at the level of the jejunum. Kidney tPO_2 and flow was measured in the left kidney at the level of the cortico-medullary junction. Muscle tPO_2 and flow was measured in the left vastus intermedius muscle at a depth of 10 mm. Data for tissue pO_2 and flows are shown in Study 1 Appendices 12-14 (p51-53).

Major Finding 1:

3% NaCl ALM led to a significant increase in local gut pO_2 and blood flow and more balanced supply/demand index compared to saline controls and Hextend-treated animals.

3% NaCl ALM bolus led to a significant increase in local gut pO_2 and blood flow at the level of the jejunum compared to 3% saline alone (Appendix 12, p52). Interestingly, after commencement of Phase 2 0.9% NaCl ALM drip, the tissue pO_2 decreased over the next 120 min period but flow continued to increase. Since tissue pO_2 is a ratio of supply and demand, a decreasing pO_2 can only occur if demand is lowered. The next 2 hour 'drip' resulted in smaller decreases in tissue pO_2 for decreases in flow indicating a more constant supply and demand index and stabilization around baseline levels. To summarize, the supply demand index increased during the Phase 1 bolus, lowered during early Phase 2 (first 2 hours) and stabilized over the remaining late Phase 2 period. The supply demand index in 3% NaCl controls was relatively constant relative to baseline and similar to Shams over Phase 1 and 2. Hextend resulted in a lower supply demand index from lower tissue pO2 and lower flow, which was associated with cardiac/organ failure and early death.

That 3% NaCl ALM led to higher pO_2 and flow was a significant finding because hypoperfusion of splanchnic organs is believed to be an important contributor to the development of systemic inflammation (SIRS), multiple organ failure (MOF) and death. In addition to widespread endothelium injury from lower cardiac output and systemic hypoperfusion, SIRS and MOF can be exacerbated by the ischemic gut, which releases proinflammatory factors and immune cells into the general circulation via lymph from the mesenteric nodes (bypassing portal vein and liver) at the level of the thoracic duct. Toxic gut-derived lymph thus adds to secondary organ injury and dysfunction. Our data showing that 3% NaCl ALM increase pO_2 and blood flow to the gut (jejunum) during hemorrhagic shock may prevent toxic lymph from forming from reduced ischemia-reperfusion injury. The data are consistent with our recent study on guinea-pig pressurized mesenteric artery segments where adenosine and lidocaine combined was shown to be a potent dilator compared to A or L alone (in press). The jejunum lies between the duodenum and the ileum of the small intestine and supplied by branches of the superior mesenteric artery from the abdominal aorta.

Major Finding 2:

3% NaCl ALM bolus prevented hemorrhage-induced hypoperfusion of the kidney. 0.9% NaCl ALM drip lowered metabolic demand in the kidney and gut.

3% NaCl ALM increased kidney pO_2 and flow during Phase 1 resuscitation. During early Phase 2 pO_2 fell and flow increased indicating, similar to the gut, lower supply/demand index from lower metabolic demand. From the first to third hour in Phase 2 ALM supply/demand index was relatively stable from lower pO_2 and lower flow, and after 180 min there was a large fall in flow but a constant pO_2 indicating demand must have dropped. 3% NaCl kept pO_2 relatively stable at the expense of lower flow to the kidney. The ALM results are important because hypoperfusion of the kidney occurs early after hemorrhage due the sympathetic-adrenal medulla system and activation of the renin-angiotensin-aldosterone system.

STUDY 2

Traumatic Brain Injury (TBI) ALONE (no hemorrhage)

Background: TBI has also been implicated in Gulf War Syndrome, which affects the integration of musculoskeletal, digestive, integumentary and neurosensory systems, and has been reported in around 25% of return service personnel. There is also emerging consensus in military and sports medicine of a possible link between TBI and posttraumatic stress disorder (PTSD). Development of novel pharmacotherapies for the treatment of traumatic injury in military or civilian settings has been ongoing for over 40 years. Currently none have successfully translated. 3.0% saline has shown to be effective in reducing edema and intracranial pressure (ICP) in a number of studies and there appears to be a growing trend favoring the use of hypertonic sodium solutions over mannitol in patients with TBI.

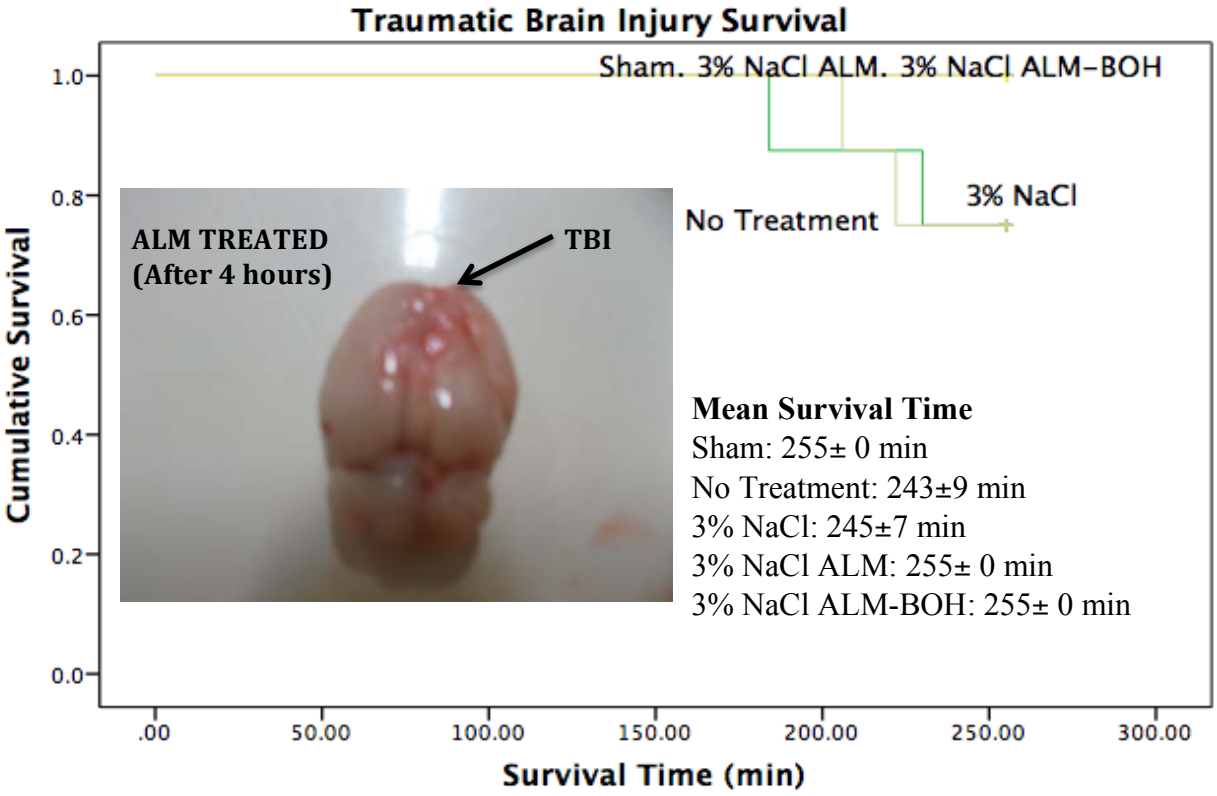
The aim of Study 2 was to compare hypertonic saline bolus and normal saline drip with 3% NaCl ALM bolus (Phase 1 60 min) and 0.9% NaCl ALM "drip" (Phase 2 = 3 hours) in a rat model of mild-to-moderate traumatic brain injury (TBI) induced by fluid percussion. The mechanisms of traumatic brain injury-associated coagulopathy are still unclear and it was hypothesized that ALM therapy would prevent TBI-associated coagulopathy in the non-bleeding patient. Beta-hydroxybutyrate, a important brain fuel and neuroprotectant during stress, was added to the ALM bolus and drip, to examine synergistic/additive beneficial effects.

A lateral model of fluid percussion injury (LFP) with mild-to-moderate force of 2.3-2.5 atm was performed according to the method of Kabadi *et al* (2010). LFP produces both focal and diffuse injury alterations in cerebral blood flow, increased permeability of the blood-brain barrier, vascular disruption and neuronal cell death. Animals were attached to Small Animal Stereotaxic Instrument with Digital Display Console (Model 940, Kopf Instruments, California, USA) and a 4mm craniotomy was trephined over the parietal region, 4 mm lateral to the sagittal suture, midway between bregma and lambda. Dental acrylic was used to secure a female Luer Loc for attachment to the fluid percussion device (HPD-1700, Dragonfly Inc., West Virginia) and a digital oscilloscope (Tektronix, Oregon, USA) recorded a fluid percussion injury of mild-to-moderate severity (2.3-2.5 atm). Treatment commenced 15 min after injury with a 0.7 ml/kg bolus (Phase 1 resuscitation) followed 60 min later by a 0.5 ml/kg/hr 'drip' for three hours (Phase 2 resuscitation). Sham animals had craniotomy and placement of Luer Loc but did not receive fluid percussion injury.

1. Survival

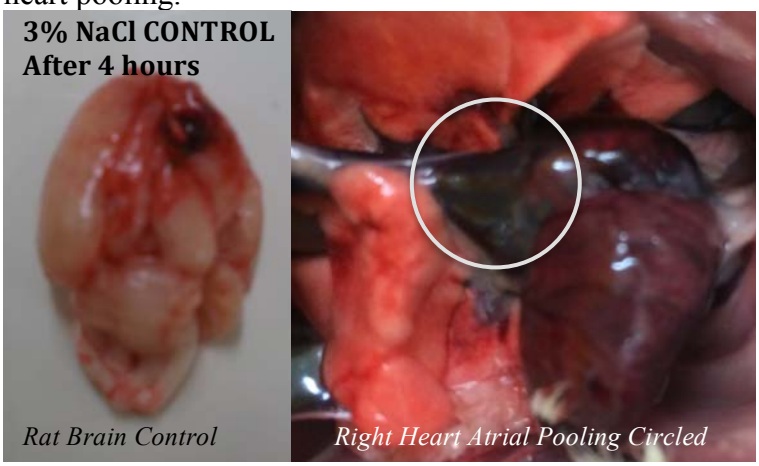
Major Finding:

ALM and ALM + beta-hydroxybutyrate therapy led to 100% survival after four hours treatment, compared to Untreated animals and Saline group with 25% mortality.



Note: Maximum possible time for survival was 255 min and set by the protocol after fluid percussion injury. Time of death was time taken from time of brain injury until MAP decreased <20 mmHg, at which point animal was considered unviable.

Non-survivors in 3% NaCl group had increased bleeding at point of TBI and cardiovascular collapse with increased complex ventricular arrhythmias (tachycardia and fibrillation) and right heart pooling.

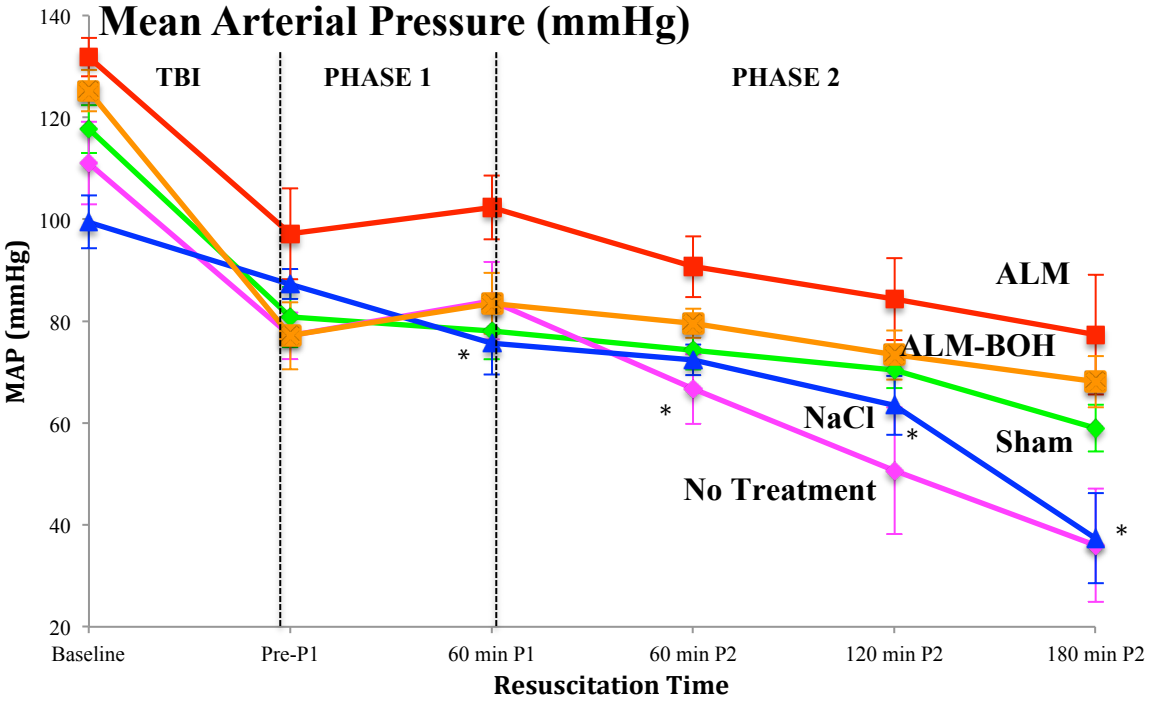


Photographs taken from animal receiving 3% NaCl bolus and 0.9% NaCl drip which died 208 min after fluid percussion injury. Increased bleeding at site of injury was associated with a severe hypocoagulopathy and right heart pooling was associated with arrhythmias and cardiovascular collapse. Right atrial pooling circled

2. Hemodynamics

Major Finding:

ALM bolus-infusion therapy improved hemodynamics after TBI with significantly increased MAP and pulse pressure compared to saline controls.



* $p<0.05$ c.f. 3% NaCl ALM

Note: Pre-P1 measurement taken 15 min after fluid percussion injury just prior to Phase 1 fluid bolus

Parameter	Time	**Sham**	**No Treatment**	**3% NaCl**	**3% NaCl ALM**	**3% NaCl ALM BOH**
Systolic Pressure (mmHg)	Pre-P1	107±7	101±6	110±5	123±11	102±7
	60 min P1	107±6	112±9	97±9*	134±8	109±2
	180 min P2	82±5	49±15*	50±13*	106±14	94±7
Diastolic Pressure (mmHg)	Pre-P1	68±5	65±4	76±4	84±8	65±7
	60 min P1	63±5	70±7	65±5	86±6	70±8
	180 min P2	48±4	29±9*	31±7*	63±11	55±4
Pulse Pressure (mmHg)	Pre-P1	39±3	36±4	34±5	39±5	38±4
	60 min P1	44±2	42±3	33±6	48±3	40±8
	180 min P2	34±2	20±6*	20±6*	43±4	39±5

* $p<0.05$ compared to 3% NaCl ALM

3. Temperature

Major Finding:

ALM treatment prevented elevations in post-traumatic body temperature after TBI compared to 3% NaCl controls.

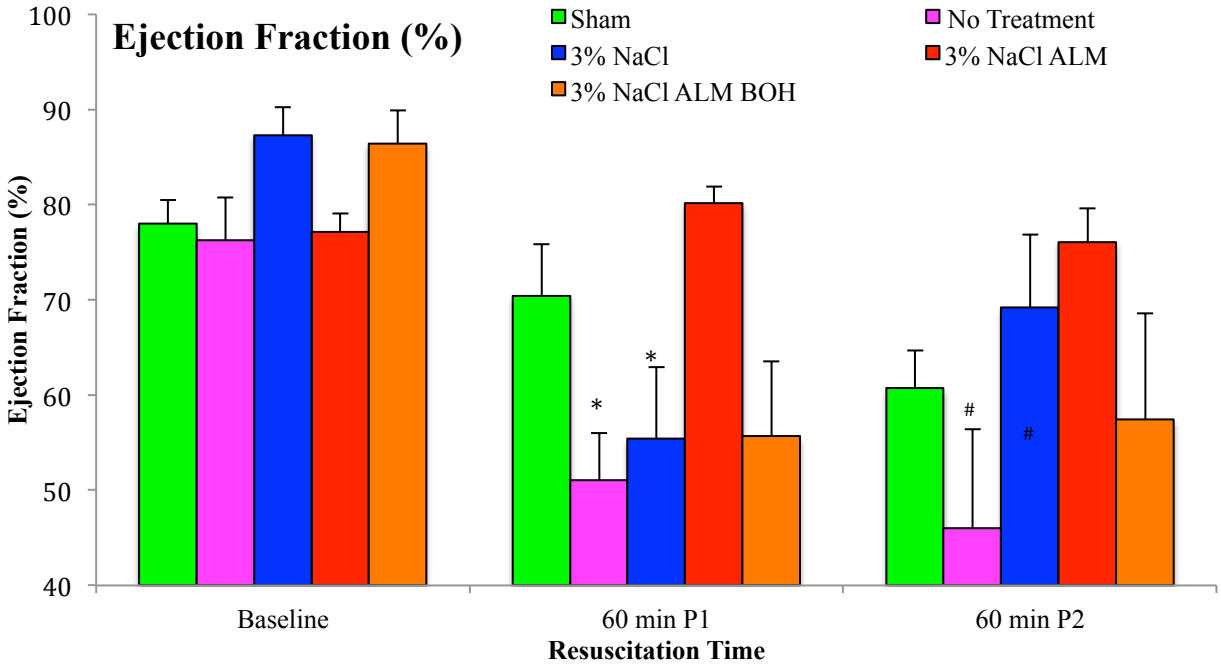
Background: Fever burden after TBI has been associated with increased secondary injury mechanisms and worse outcomes.

3% NaCl controls and untreated animals experienced temperature increases of 2-3°C after traumatic brain injury (36-38°C) with greater variability during resuscitation phases 1 and 2. In contrast, ALM bolus-infusion therapy maintained a mildly hypothermic state (33-34°C) that was stable throughout the entire monitoring period.

4. Cardiac Function

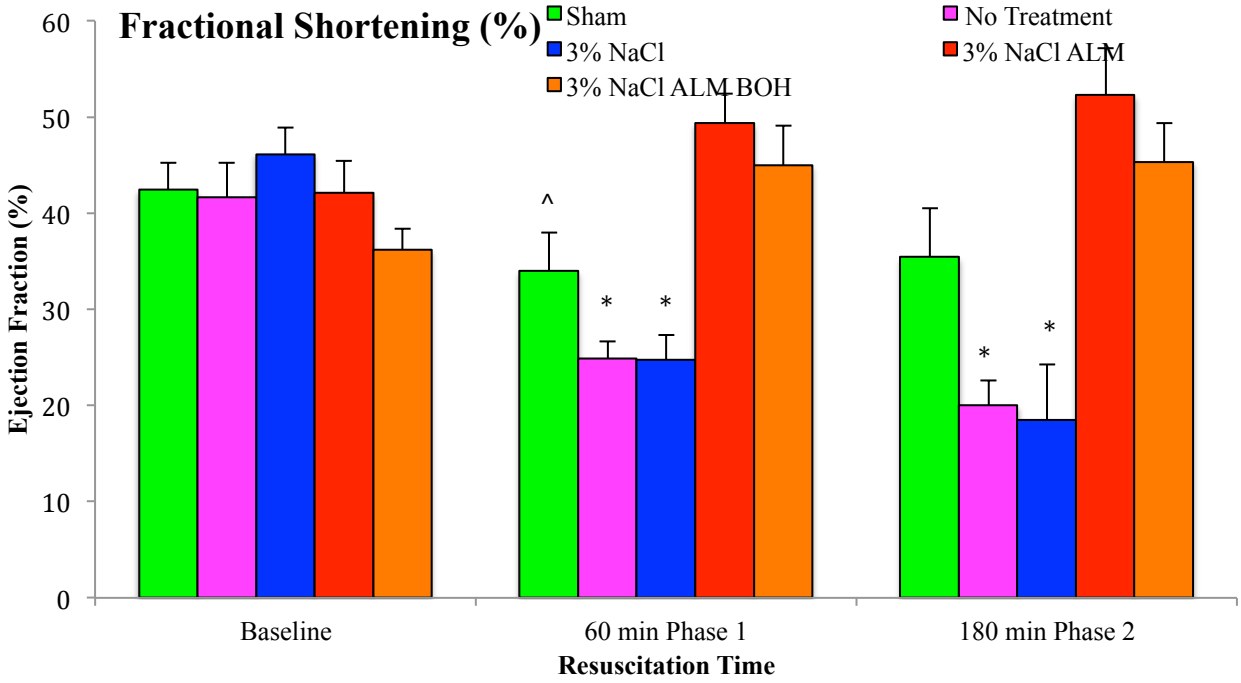
Major Finding:

> **ALM-treated animals had improved cardiac function with significantly increased ejection fraction, cardiac output and cardiac contractility, and significantly fewer arrhythmias compared to saline controls.**



* $p<0.05$ c.f. 3% NaCl ALM and 3% NaCl ALM BOH
\# $p<0.05$ c.f. Sham, 3% NaCl ALM and 3% NaCl ALM BOH

Note: Left ventricular fractional (LVF) shortening is dependent on LV preload, afterload and geometry, as well as contractility. Although widely used clinically as an index of contractility, ideally contractility is independent of load but this is very difficult to assess *in vivo*. LV contractility refers to the contractile state of the whole LV. Indices of LV contractility conventionally have been divided into isovolumic phase indices (peak positive dP/dt), ejection phase indices (systolic wall stress versus endocardial shortening), and those determined at the end of ejection (end-systolic elastance).



* $p<0.05$ c.f. 3% NaCl ALM and 3% NaCl ALM BOH
^ $p<0.05$ c.f. Sham, 3% NaCl ALM and 3% NaCl ALM BOH

Group	Time	Heart Rate (bpm)	Stroke Volume (ml)	Cardiac Output (ml/min)
Baseline	Sham	358±7	0.21±0.06	74.0±20.8
	No Treatment	335±16	0.28±0.06	90.1±18.9
	3% NaCl	306±15	0.21±0.06	61.9±16.1
	3% NaCl ALM	369±10	0.20±0.07	77.8±27.7
	3% NaCl ALM BOH	330±10	0.29±0.04	94.7±12.6
60 min Phase 1	Sham	301±16	0.15±0.03	41.6±9.2
	No Treatment	339±25	0.15±0.03	47.4±11.6
	3% NaCl	297±11	0.25±0.09	77.0±30.5
	3% NaCl ALM	326±11	0.24±0.09	76.3±27.6
	3% NaCl ALM BOH	272±8	0.21±0.03	56.9±7.6
180 min Phase 2	Sham	276±13	0.10±0.02	31.8±4.10
	No Treatment (n=6)	305±11	0.10±0.02	29.2±6.7
	3% NaCl (n=6)	324±14	0.07±0.03	13.6±3.9*
	3% NaCl ALM	296±11	0.17±0.02	49.9±7.6
	3% NaCl ALM BOH	271±14	0.13±0.03	34.0±7.1

* $p<0.05$ c.f. 3% NaCl ALM

In addition to improving cardiac function ALM bolus-infusion therapy also reduced pulmonary edema compared to saline controls (lung wet:dry ratio 4.92±0.08 vs. 5.30±0.14) and reduced neutrophil activation in the lung (myeloperoxidase assay).

5. Coagulopathy

Major Finding 1:

ALM treatment corrected PT and ROTEM-assessed traumatic brain injury-associated coagulopathy compared to 3% saline controls.

- **Controls showed a worsening hypocoagulopathy after Phase 2 resuscitation.**
- **ALM correction was associated with 1.5 times higher plasma fibrinogen levels compared to 3% NaCl controls.**

Group	PT (sec)		aPTT sec)		Fibrinogen (g/dL)	
	Phase 1	Phase 2	Phase 1	Phase 2	Phase 1	Phase 2
Baseline	15±0.3[#]		31±3[#]		2.40±0.08	
Sham	19±2	31±10	161±20	142±31	1.93±0.15[†]	1.81±0.30
No Treatment	27±6	72±26	154±21	113±29	1.88±0.20	1.24±0.22*
3% NaCl	28±6	113±26*	150±25	172±19	2.33±0.19	0.96±0.20*
3%NaCl ALM	18±0.8	22±2	142±42	160±11	2.43±0.03	2.41±0.11
3% NaCl ALM BOH	19±0.8	21±2	200±0	191±10	2.23±0.6	2.30±0.06

[#] $p<0.05$ compared to all groups
* $p<0.05$ compared to Baseline, 3% NaCl ALM, and 3% NaCl ALM BOH
[†] $p<0.05$ compared to ALM

Representative TEMograms for each group from Phase 1 and Phase 2 resuscitation are shown over (p29).

Major Finding 2:

ALM and ALM with beta-hydroxybutyrate better preserved platelet function compared to Untreated animals and 3% NaCl controls.

Collagen-induced platelet aggregation was significantly higher in animals treated with ALM and ALM-beta-hydroxybutyrate compared to untreated animals and saline controls.

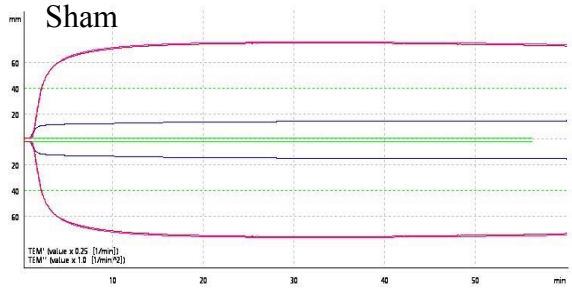
Collagen-Induced Platelet Aggregation			
Group	Primary Slope (°)	Maximum Aggregation (%)	Disaggregation (%)
Sham	28±10	42±15	9±6
No Treatment	1±0.8[#]	2±1[#]	-
3% NaCl	6±4*	9±5*	1±0.5
3% NaCl ALM	51±32	48±25	13±6
3% NaCl ALM BOH	39±15	42±15	6±4

* $p<0.05$ c.f. 3% NaCl ALM and 3% NaCl ALM-BOH
[#] $p<0.05$ c.f. Sham, 3% NaCl ALM and 3% NaCl ALM-BOH

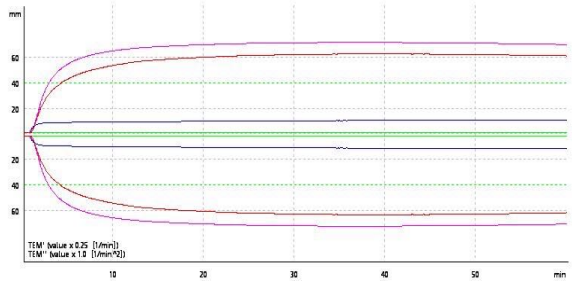
Representative TEMograms

PHASE 1 RESUSCITATION

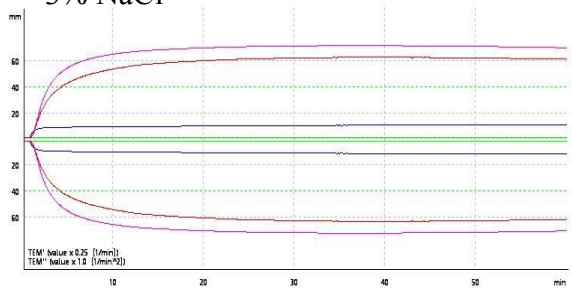
Sham



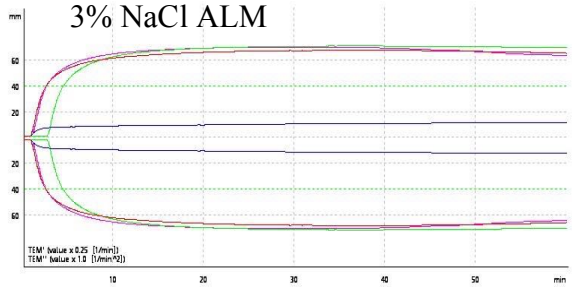
No Treatment



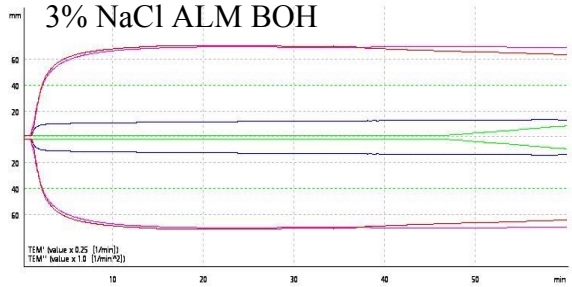
3% NaCl



3% NaCl ALM

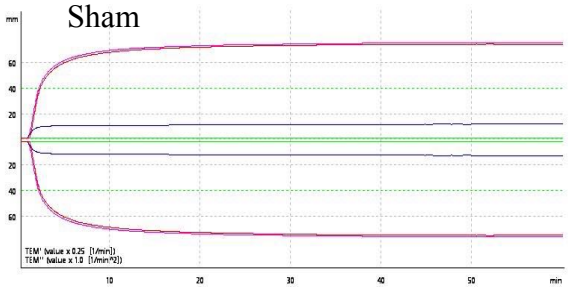


3% NaCl ALM BOH

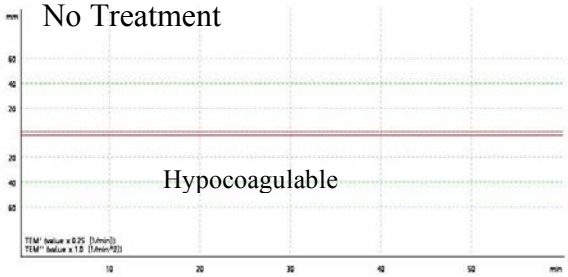


PHASE 2 RESUSCITATION

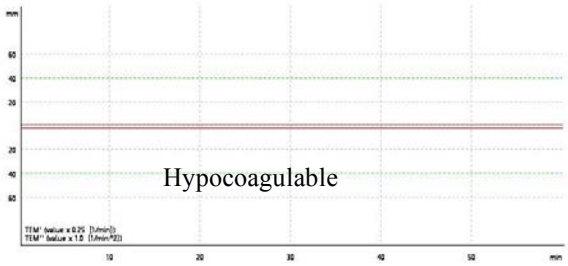
Sham



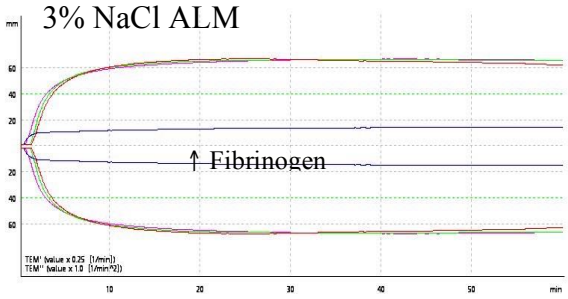
No Treatment



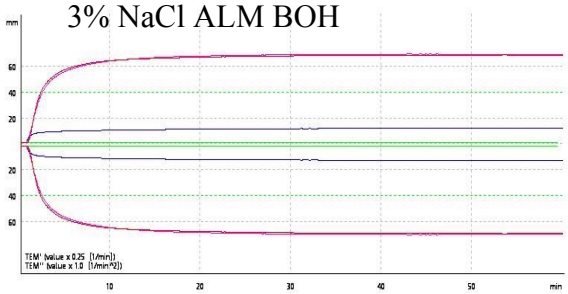
3% NaCl



3% NaCl ALM



3% NaCl ALM BOH



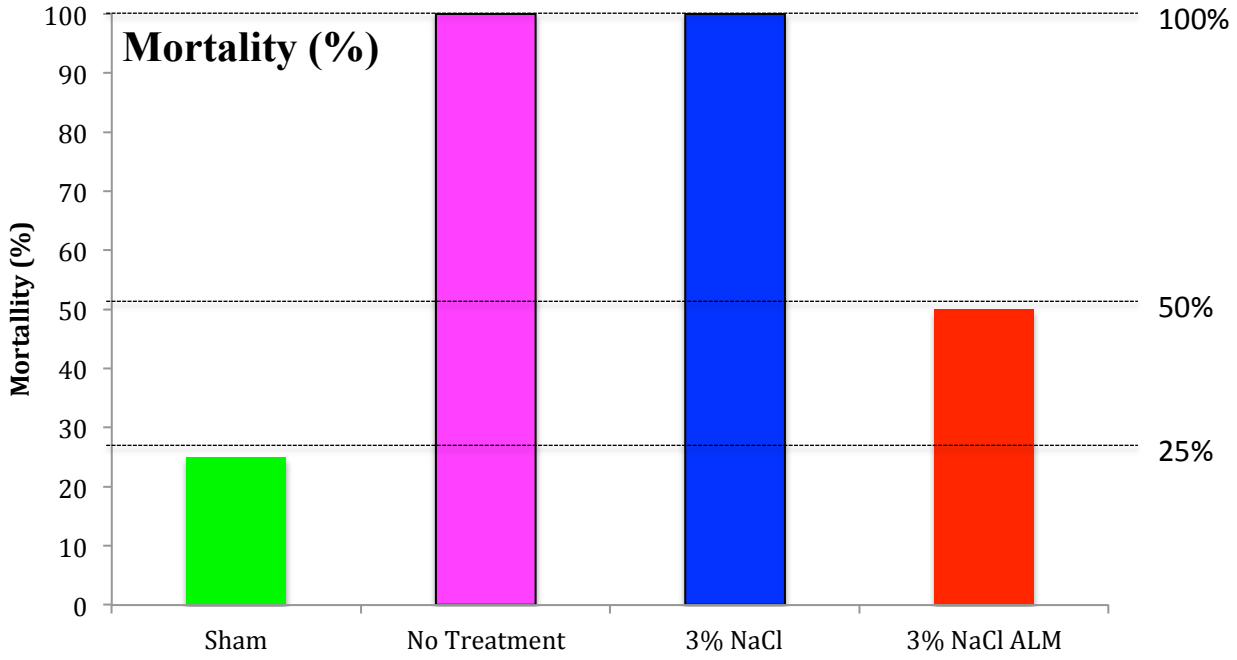
STUDY 3

Lethal Model of TBI + 'Non-Compressible' Internal Bleeding

In the final study animals received a mild traumatic brain injury (2.1 atm fluid percussion) followed three minutes later by 50% liver resection to induce uncontrolled hemorrhage. Fifteen minutes following induction of TBI 0.7 ml/kg bolus was administered (Phase 1 resuscitation). Sixty minutes after bolus, Phase 2 resuscitation commenced comprising three hour 0.5 ml/kg/hr drip.

1. Survival Outcomes

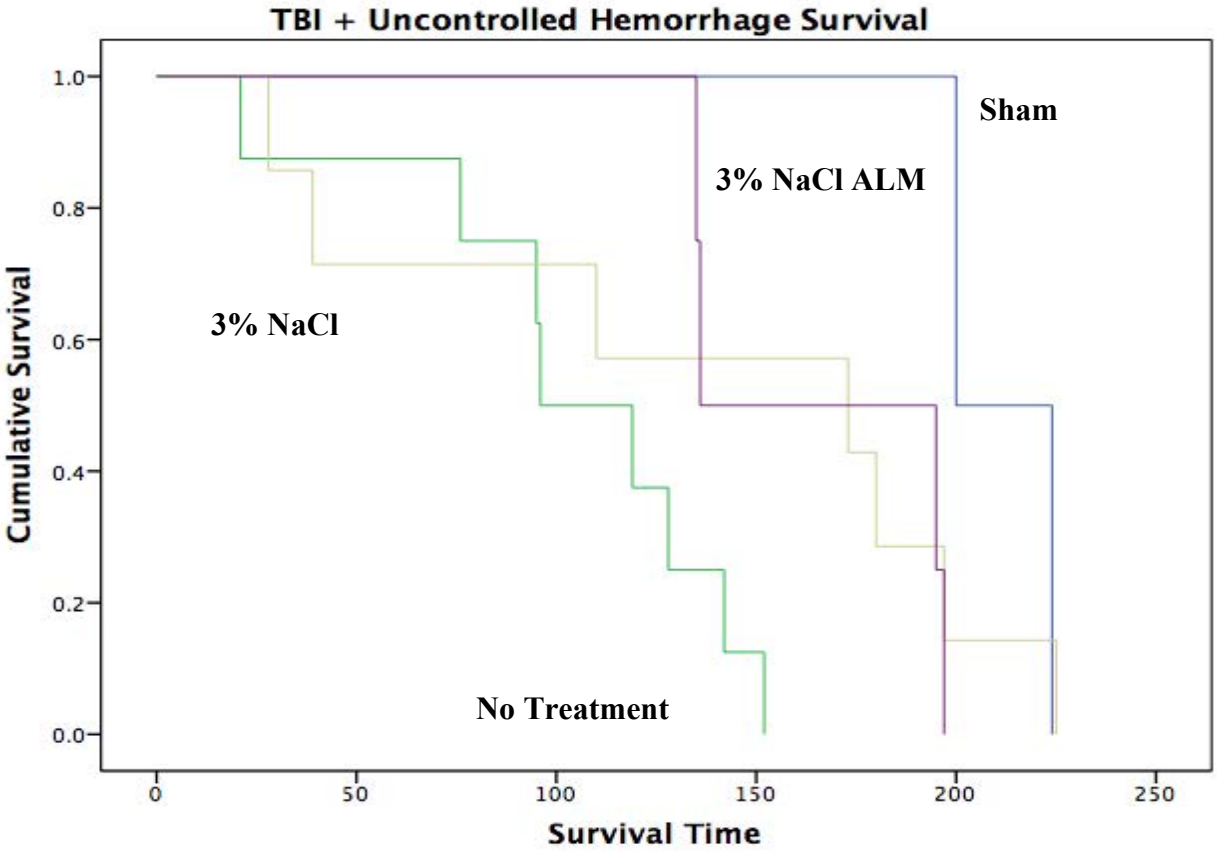
ALM bolus-infusion treatment reduced mortality by 50% and prolonged survival time compared to saline controls in the lethal two-hit model of traumatic brain injury and uncontrolled hemorrhage.



NB: Sham animals underwent craniotomy and placement of Luer Loc followed by single laparatomy *without fluid percussion injury or liver resection and bleeding*. Sham animals received identical treatment to 3% NaCl controls (0.7 ml/kg 3% NaCl bolus Phase 1, 0.5 ml/kg/hr 0.9% NaCl drip Phase 2). No Treatment animals underwent fluid percussion injury followed by liver resection and uncontrolled bleeding *without fluid resuscitation*.

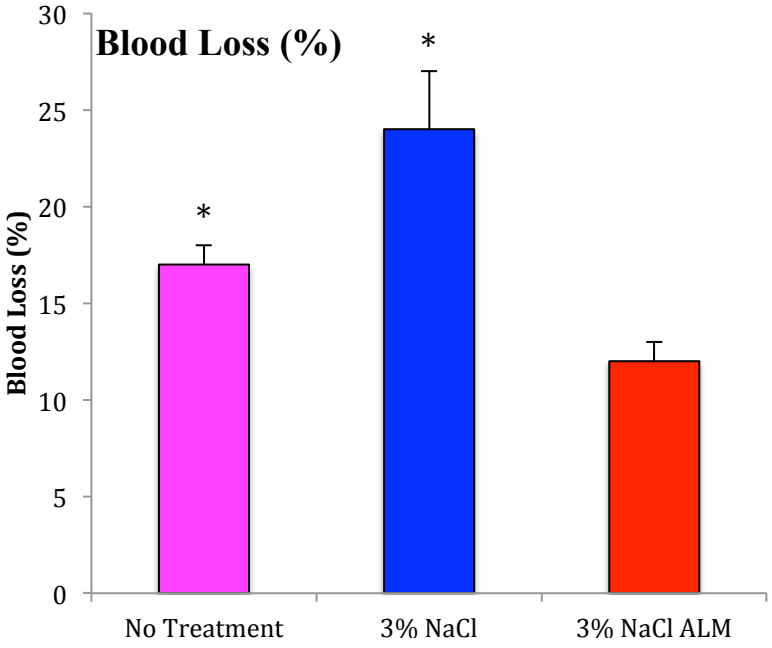
Mean Survival Time (min from TBI)	
Sham	212±12
No Treatment	104±15
3% NaCl	136±30
3% NaCl ALM	166±17

Note: Maximum possible time for survival was 255 min and set by the protocol after fluid percussion injury.



2. Non-compressible Blood Loss

ALM bolus-infusion therapy led to <u>50% less blood loss</u> compared to saline controls

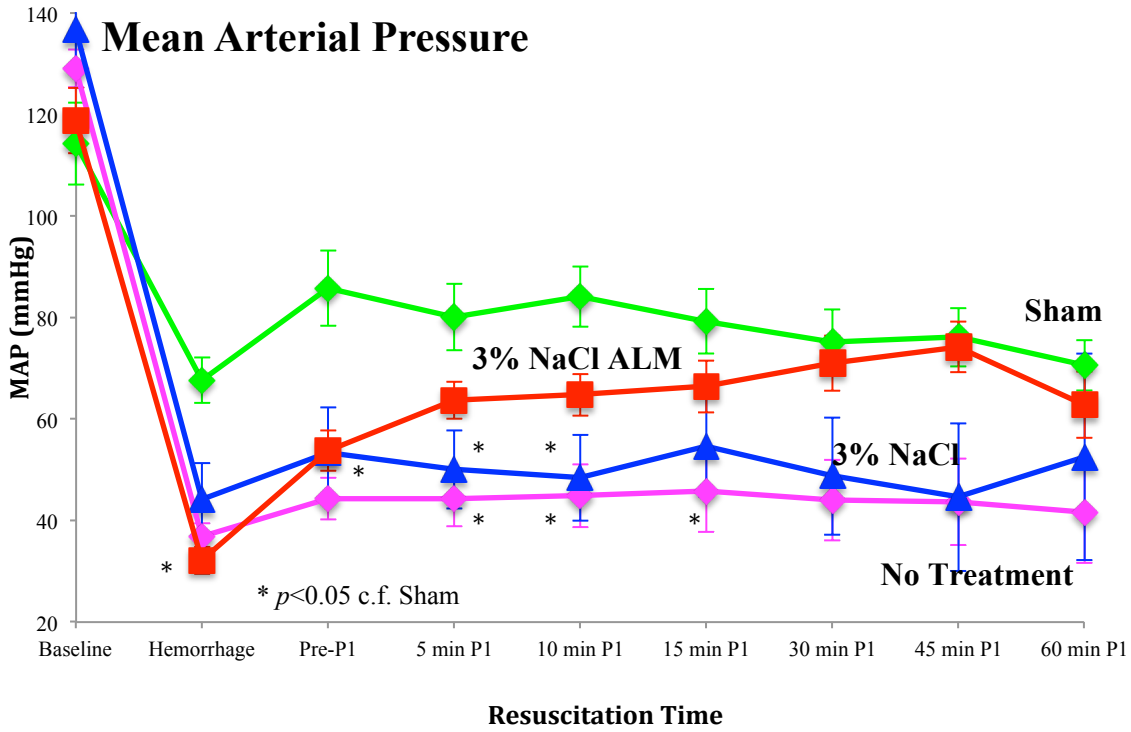


* $p<0.05$ c.f. 3% NaCl ALM

Blood loss, expressed as a percentage of total blood volume (calculated from [(0.06 x body weight + 0.77]) was estimated at time of death/sacrifice by mopping up clots and loose blood in the abdominal cavity with pre-weighed gauze.

3. Hemodynamics

3% NaCl ALM bolus resuscitated mean arterial pressure following combined traumatic brain injury and uncontrolled hemorrhage.



Following 60 min Phase 2 resuscitation MAP (mmHg) in Shams, Untreated, Saline, and ALM group was 74±7, 15±6, 26±11, and 41±9, respectively. In contrast to No Treatment group and Saline controls, ALM-treated animals maintained a MAP that was not significantly different from Shams from 120-180 min Phase 2.

4. Coagulopathy

Major Finding 1:

- **3% NaCl ALM bolus increased fibrinogen, restored clot amplitudes, and corrected surgery-associated INTEM coagulopathy.**
- **3% NaCl controls were hypocoagulable after Phase 1 resuscitation with decreased clot initiation and stability, reduced fibrinogen, and no clot initiation on INTEM.**

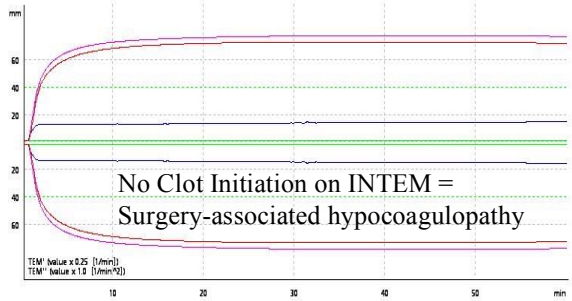
Major Finding 2:

- **ALM drip therapy preserved EXTEM, APTEM and FIBTEM clot initiation, propagation and amplitudes, with significantly improved coagulation parameters compared to all other groups after Phase 2 resuscitation.**
- **Coagulopathy progressively worsened in saline controls after Phase 2 resuscitation.**

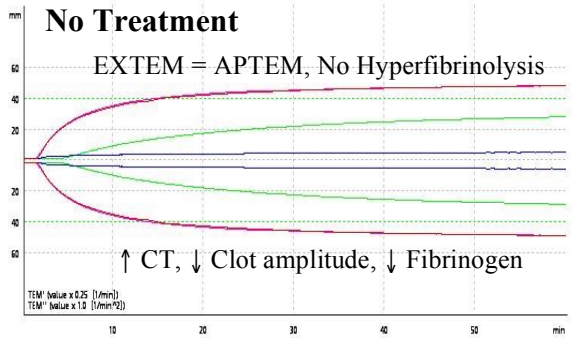
Representative TEMograms

PHASE 1 RESUSCITATION

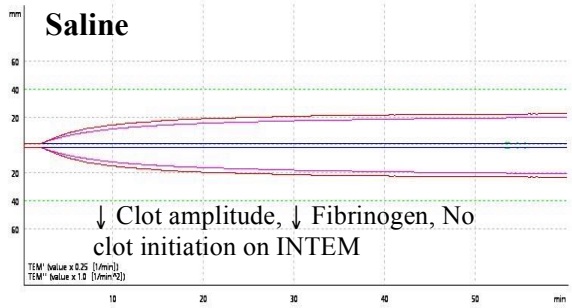
Sham



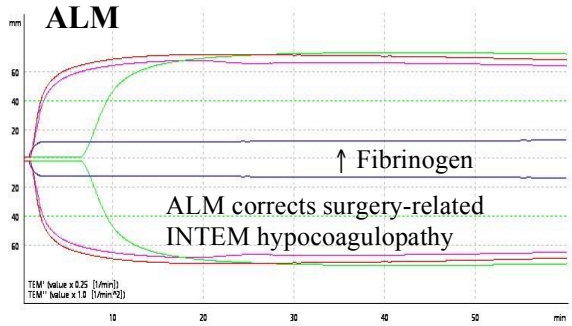
No Treatment



Saline

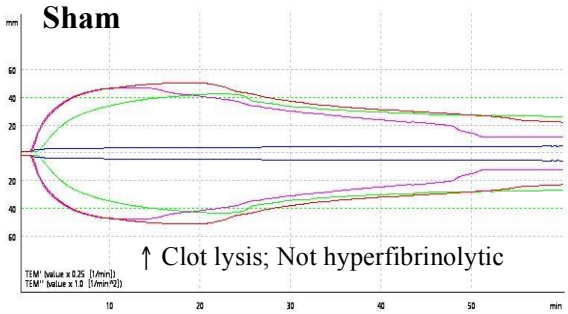


ALM

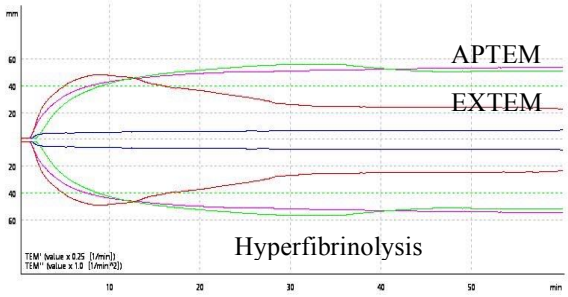


PHASE 2 RESUSCITATION

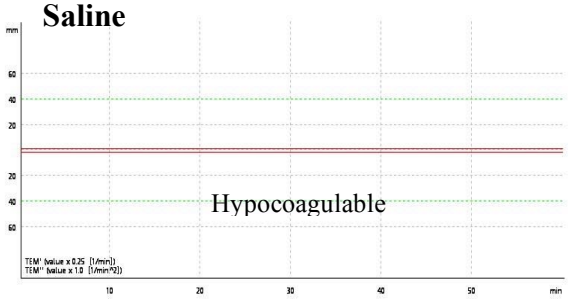
Sham



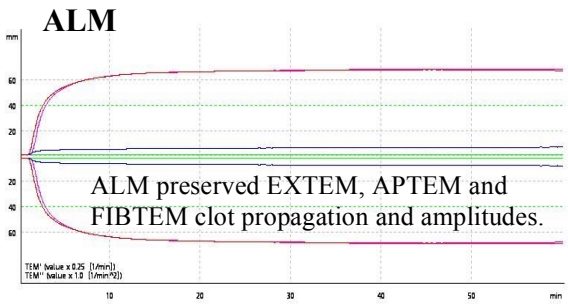
No Treatment



Saline



ALM



3. ACCOMPLISHMENTS continued:

d. Unexpected achievements and significant results

A totally unexpected result was that small-volume 3% NaCl ALM bolus and 0.9% NaCl ALM 'drip' (3 to 4 hours) reduced truncal haemorrhage by up to 60% after hemorrhagic shock (60% liver resection) (discussed above). This major finding may be significant in the advancement of far-forward medicine as they provide the SOF combat medic/corpsman with a new small-volume resuscitation strategy to treat shock in severely injured combatants with suspected internal bleeding. ALM resuscitation therapy acted like a "pharmacological tourniquet for uncontrolled internal bleeding".

e. What opportunities for training and professional development has the project provided?

"*Nothing to Report*."

f. How were the results disseminated to communities of interest?

We have not released our significant findings to the public or media as of Feb 29, 2016 but will do so after seeking approvals from our contract liaison Cheryl A. Lowery U.S. Army Medical Research Acquisition Activity, (Cheryl.a.lowery8.civ@mail.mil), Katie Boggs (Boggs, Katie L CTR USARMY MEDCOM USAMRMC (US) (katie.l.boggs.ctr@mail.mil) and USSOCOM project manager Mr Benjamin Chitty "Chitty, Benjamin L CIV USSOCOM HQ" (Benjamin.Chitty@socom.mil).

g. What do you plan to do during the next reporting period to accomplish the goals?

"*Nothing to Report*." We have submitted new studies to continue to translate new fluid therapy into the battlefield.

a. **What was the impact on other disciplines?**

"*Nothing to Report*."

b. **What was the impact on technology transfer?**

"*Nothing to Report*."

h. What was the impact on society beyond science and technology?

"*Nothing to Report*."

3. CHANGES/PROBLEMS:

a. Changes in approach and reasons for change

"Nothing to Report"

b. Actual or anticipated problems or delays and actions or plans to resolve them

The following minor problems and delays were resolved. A 3 months extension was requested and allowed.

- Pilot TBI animals in Study 2 also showed that Sham animals (craniotomy alone – no percussion injury) had decreased MAP and did not survive total surgery + experiment time (365 min from time of injury. Therefore the 'drip' Phase 2 period was reduced from 4 to 3 hours. The same timeline was used for the two-hit model Study 3 (TBI+ liver uncontrolled hemorrhage). It is believed that the reduced survivability was in part due to the detrimental effect of long-term Thiobarb anaesthesia and mechanical ventilation
  .
- We reduced assessing platelet function from 4 agonists to two to conserve blood in the three trauma models. Agonists ADP and collagen were selected as these are the most widely used in the literature to assess platelet function.

- 3 x ventilators, 4 x syringe pumps, 1 x ROTEM channel, and pO2 electrode on blood gas analyser broke down in the course of the project which led to delays in experimental work being carried out.

c. Changes that had a significant impact on expenditures

No Changes

d. Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

No Changes

e. Significant changes in use or care of human subjects

Not Applicable

f. Significant changes in use or care of vertebrate animals.

None

g. Significant changes in use of biohazards and/or select agents

None

5. PRODUCTS:

i) Publications, conference papers, and presentations:

Three abstracts have been submitted to MHSRS (Fort Lauderdale) for 2016 Military Meeting. These abstracts as well as three additional publications are currently being written up for full publications in high profile referred journals (e.g. J Trauma, Nature, Critical Care, Critical Care Medicine). Abstracts 1 to 3 summarize the major findings for STUDY 1 to 3 above.

> **ABSTRACT 1**: Small-volume 3.0% NaCl ALM bolus and 'drip' reduces non-compressible hemorrhage by 60%, corrects coagulopathy and reduces inflammation in a rat model of truncal bleeding and shock.
>
> **ABSTRACT 2:** Small-volume 3.0% NaCl ALM therapy increases survival, corrects coagulopathy and prevents neurogenic cardiac depression after mild TBI in rats: a potential new therapy.
>
> **ABSTRACT 3:** Small-volume 3.0% NaCl ALM resuscitation therapy leads to 50% survival and 50% less internal bleeding in a lethal rat model of combined TBI and truncal hemorrhage

MANUSCRIPT ABSTRACT 1:

Small-volume 3.0% NaCl ALM bolus and 'drip' reduces non-compressible hemorrhage by 60%, corrects coagulopathy and reduces inflammation in a rat model of truncal bleeding and shock.

Background: Noncompressible torso hemorrhage is the leading cause of potentially survivable trauma in far-forward combat environments. Our aim was to examine the effect of small-volume 3% NaCl adenosine, lidocaine and Mg^{2+} (ALM) bolus and 0.9% NaCl/ALM 'drip' on hypotensive resuscitation, hemostatic function and survivablity in the rat model of uncontrolled hepatic hemorrhage and shock.

Methods: Male Sprague-Dawley rats (300-450g) were anesthetized (thiobarbital) and randomly assigned to one of five groups (n=8 each): 1) Sham, 2) No Treatment, 3) 3% NaCl controls, 4) 3% NaCl ALM and 5) Hextend®. Animals were ventilated, instrumented and hemorrhagic shock was induced by 55% liver resection (60% left lateral lobe/50% medial lobe). After 15 min shock, a single bolus (0.7 ml/kg) was injected IV and after 60 min (Phase 1), 0.9% NaCl ± ALM stabilization 'drip' (0.5 ml/kg/hour) was commenced and continued for 4 hours (Phase 2). Coagulopathy and fibrinolysis were assessed using PT, aPTT, ROTEM and STAGO, and inflammatory status using ELIZA kits. Separate groups (double laparotomy) were required for gut, kidney and muscle pO_2 and flow (Optronix, Oxford). Cardiac function was assessed *in vivo* using 2-D-echocardiography. Internal blood loss was estimated in the peritoneal cavity from collecting clots and blood fluids with a gauze at the time of sacrifice and weighed

Results: Mortality for Shams (no resection) was 0% (25%); No Treatment, 87.5% (100%); 3% NaCl, 37.5% (75%); 3% NaCl ALM, 0% (25%), and Hextend, 87.5% (100%) (double laparotomy in parentheses). Hextend-treated animals died early during the first 20 min of Phase 2. ALM bolus during Phase 1 led to 2.4 fold higher cardiac output, 1.7 times contractility,

improved hemodynamics, fewer arrhythmias, correction of PT, aPTT and ROTEM clot times, amplitude and lysis indices, higher fibrinogen, higher body temperature and lower IL-1 alpha and beta, TnF-alpha, IL-6, RANTES and IF-gamma compared to untreated animals, 3% NaCl controls or Hextend rats. ALM bolus also led to increased tissue pO_2 and flow in the gut and kidney, and in Phase 2 tissue pO_2 decreased but flow continued to rise, indicating lower metabolic demand in both organs compared to controls. During phase 2, there was a worsening of CO, contractility, systemic inflammation and coagulopathy in all groups except ALM treated-animals. ALM therapy had significantly improved platelet function, attenuated systemic inflammation, oxidative stress, pulmonary edema, reduced liver injury (AST) resulting in up to 60% less bleeding over 5 hours compared to 3% NaCl controls, and 75% less bleeding than Hextend-treated rats.

Conclusions: Small-volume 3% NaCl ALM therapy significantly reduced mortality and internal bleeding compared to 3% NaCl controls or Hextend-treated rats. Reduced mortality and blood loss may arise from ALM's ability to correct coagulopathy, blunt systemic inflammation and improve Central-CardioVascular-Endothelium coupling to meet changing tissue O_2 needs. ALM resuscitation may translate and provide SOF combat medic/corpsman with a new way to decrease non-compressible hemorrhage and improve warfighter survivability in far-forward environments.

Key Points:

1. Small-volume 3% NaCl ALM bolus significantly improved survivability and reduced non-compressible truncal blood loss by up to 60% compared to 3% NaCl controls.

2. Increased survivability and less internal blood loss may have arisen from ALM's ability to improve cardiac and hemodynamic function, correct coagulopathy, reduce systemic inflammation, improve platelet function, and increase gut tissue pO_2 and blood flow.

3. Hextend administered as a bolus according to TCCC guidelines promoted internal bleeding, systemic coagulopathy, inflammation, organ failure and early death (100% mortality).

MANUSCRIPT ABSTRACT 2

Small-volume 3.0% NaCl ALM therapy increases survival, corrects coagulopathy and prevents neurogenic cardiac depression after mild TBI in rats: a potential new therapy.

Background: Traumatic brain injury (TBI) is a major cause of mortality and morbidity on the battlefield, in civilian war zones and following terrorist attacks. Despite decades of research, new frontline drug therapies are urgently required to reduce or prevent the development of secondary brain injury. We have developed a new small-volume 3% NaCl adenosine, lidocaine and Mg^{2+} (ALM) bolus and 0.9% NaCl/ALM 'drip' therapy for uncontrolled truncal blood loss and shock. Our aim was to examine the effect of the ALM fluid therapy on survivability and secondary 'hit' complications in a rat fluid-percussion model of mild TBI.

Methods: Male Sprague-Dawley rats (300-450g) were anesthetized (thiobarbital) and randomly assigned to one of five groups (n=8 each): 1) Sham, 2) No Treatment (NT), 3) 3% NaCl, 4) 3% NaCl ALM, and 5) 3% NaCl ALM/BHB (beta-hydroxybutyrate). Animals were ventilated and

surgically instrumented with femoral arterial and venous catheters. TBI of mild-to-moderate severity (2.3-2.5 atm) was produced in the right parietal region (~4 mm lateral to the sagittal suture) using a fluid-percussion device (Dragonfly, Inc). Shams had craniotomy/luer-lock but no TBI. After 15 min shock, a single bolus treatment (0.7 ml/kg) was injected IV and after 60 min (Phase 1), 0.9% NaCl ± ALM stabilization 'drip' (0.5 ml/kg/hour) was commenced and continued for 3 hours (Phase 2). Coagulopathy and fibrinogen were assessed using PT, aPTT, ROTEM and STAGO. Cardiac function was assessed using 2-D-echocardiography.

Results: Mortality in Shams was 0%; NT, 25% (243 min); 3% NaCl, 25% (245 min); 3% NaCl ALM, 0%; and 3% NaCl ALM/BHB 0% (mean time of death in parentheses). MAP at the end of Phase 1 (60 min) was 78±5, 84±8, 76±6, 102±6 and 83±6 mmHg, and after Phase 2, was 59±5, 36±11, 37±9, 77± 12 and 68±5 mmHg for Shams, NT, 3% NaCl, 3% NaCl ALM, 3% NaCl ALM/BHB respectively. At 60 min, the 3% NaCl group had similar cardiac output (CO), stroke volume and heart rate but significantly lower LV contractility (50%) and higher body temperature (35 vs 33.5°C) compared to 3% NaCl ALM group. During Phase 2, 3% NaCl controls declined functionally, and after 3 hours had 60-70% falls in CO, stroke volume and contractility, right heart dilatation, severe arrhythmias (VT/VF) and could not form a viable clot. In direct contrast, ALM-treated animals had significantly higher CO and contractility at lower heart rates, no arrhythmias, near-full correction of coagulation parameters and twofold higher plasma fibrinogen. Overall 3% NaCl treatment was equivalent to No Treatment. BHB added to 3% NaCl ALM showed little or no improvement.

Conclusions: We conclude that small-volume 3% NaCl ALM and 0.9% NaCl/ALM 'drip' increased survivability after TBI, prevented neurogenic cardiac depression, right heart enlargement and corrected coagulopathy compared to 3% NaCl controls. Further mechanistic studies and post-injury cognition tests are required. The new ALM frontline resuscitation therapy may improve the capability of SOF combat medic/corpsman to treat combatants with suspected TBI in far-forward environments.

Key Points:

1. Diagnosing closed-head concussions in the field is challenging, and there is no safe or effective drug therapy to treat suspected TBI and secondary damage.

2. Small-volume 3.0% NaCl ALM single-bolus IV (Phase 1) and 'drip' (Phase 2) increased survival, prevented neurogenic cardiac depression and corrected coagulopathy after mild-TBI in the rat fluid percussion model.

3. ALM one-two resuscitation therapy may improve the capability of combat medics in far-forward environments to triage and treat the severely wounded with suspected TBI.

MANUSCRIPT ABSTRACT 3

Small-volume 3.0% NaCl ALM resuscitation therapy leads to 50% survival and 50% less internal bleeding in a lethal rat model of combined TBI and truncal hemorrhage

Background: In prehospital military and civilian environments, traumatic brain injury (TBI) and non-compressible truncal hemorrhage is highly lethal. We have developed a new small-volume

3% NaCl adenosine, lidocaine and Mg^{2+} (ALM) bolus and 0.9% NaCl/ALM 'drip' for uncontrolled truncal blood loss and shock, and for treating TBI alone. Our aim was to examine the effect of the ALM fluid therapy on survivability, cardiac function, coagulopathy and internal blood loss after TBI and hepatic resection in a lethal rat model.

Methods: Male Sprague-Dawley rats (300-450g) were anesthetized (thiobarbital) and randomly assigned to one of four groups (n=8 each): 1) Sham, 2) No Treatment, 3) 3% NaCl controls, and 4) 3% NaCl ALM. Animals were ventilated, instrumented and catheters placed in femoral arteries and veins. Mild TBI (2.1 atm) was produced in the right parietal region (~4 mm lateral to the sagittal suture) using a fluid-percussion device (Dragonfly, Inc). Immediately after TBI, hemorrhagic shock was induced by ~43% liver resection (50% left lateral lobe/35% medial lobe). Shams had laparotomy and craniotomy/luer-lock placement but no TBI or liver resection. Fifteen minutes after initiation of TBI, a single bolus (0.7 ml/kg) was injected IV and after 60 min (Phase 1), 0.9% NaCl ± ALM stabilization 'drip' (0.5 ml/kg/hour) was commenced and continued for 3 hours (Phase 2). Total treatment time 240 min. Coagulopathy and fibrinolysis were assessed using ROTEM. Cardiac function was assessed *in vivo* using 2-D-echocardiography. Internal blood loss was estimated in the peritoneal cavity from collecting clots and blood fluids with a gauze at the time of death/sacrifice and weighed.

Results: Mortality in Shams was 25% (212 min); No Treatment, 100% (104 min); 3% NaCl, 100% (136 min); and 3% NaCl ALM, 50% (166 min) (mean time of death in parentheses). Death was associated with progressive decompensated shock and cardiovascular collapse. At 60 min Phase 1, MAP for Shams, No Treatment, 3% NaCl and 3% NaCl ALM-treated animals was 71±5 mmHg, 42±10, 53±20, 63±7 at 60 min (n=8 each group). At the end of Phase 2, MAP in ALM survivors was 40±1 mmHg (n=4). Estimated internal blood loss for No Treatment, 3% NaCl and 3% NaCl ALM was 17±1%, 24±3% and 12±1% ($p<0.05$). At 60 min Phase 1, ALM-treated animals had improved cardiac function and reduced arrhythmias. Untreated animals and controls developed a profound hypocoagulopathy, which was corrected in ALM-treated animals.

Conclusions: In a lethal rat model of combined TBI and internal hemorrhage, small-volume 3.0% NaCl ALM bolus and 0.9% NaCl ALM 'drip' led to 50% survival, greater hemodynamic stability, corrected hypocoagulopathy and 50% less blood loss compared to controls. 100% of controls died. Two shams (laparotomy/craniotomy, no TBI or bleed) died indicating the trauma of surgery contributed 25% to the lethality of the model. Small-volume ALM fluid therapy may have wide applications for SOF medics/corpsman in far forward environments.

Key Points:

1) TBI in the military setting is often complicated by secondary hemorrhage and shock from hostile fire, bomb blasts and shrapnel.

2) Small-volume 3.0% NaCl ALM IV bolus (Phase 1) and 0.9% NaCl ALM 3 hour 'drip' stabilization (Phase 2) resulted in 50% survival, 50% less blood loss and correction of coagulopathy in a lethal rat model of combined TBI and truncal hemorrhage. 100% of 3% NaCl controls and No Treatment died early after Phase 1. Two Shams (surgery but no bleed or TBI) died (25% mortality)

3) Small-volume ALM fluid therapy may have wide field applications for SOF medics/corpsman in the treatment of internal bleeding and shock with or without suspected TBI, and after the highly lethal combination of TBI and non-compressible internal hemorrhage.

ii) Website(s) or other Internet site(s):

"Nothing to Report".

iii) Technologies or techniques:

"Nothing to Report".

iv) Inventions, patent applications, and/or licences:

"Nothing to Report".

v) Other Products:

"Nothing to Report".

6. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

i) What individuals have worked on the project:

As per original submission PI Professor Geoffrey Dobson and Research Associate Hayley Letson have both worked 16 full-time calendar months on this project. No other participants.

ii) Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

No change.

iii) What other organizations were involved as partners?

No other organizations were involved.

APPENDIX 1
Uncontrolled 'Non-Compressible' Hemorrhage and Shock
Acid-Base Balance

Parameter	Time	Sham	No Treatment	3% NaCl	3% NaCl ALM	Hextend
pH	Baseline	7.40±0.02	7.35±0.05	7.36±0.03	7.42±0.02	7.35±0.02
	60 min P1	7.26±0.04	7.20±0.08	7.17±0.05	7.30±0.02	7.11±0.06#
	60 min P2	7.23±0.02	7.06±0.11#	7.13±0.04	7.27±0.01	7.06±0.05#
	120 min P2	7.24±0.02	7.15±0.05	7.05±0.05†	7.21±0.02	6.97±0.05¶
	180 min P2	7.22±0.01	7.04±0.05*	7.00±0.06†	7.16±0.04	6.98±0.02*
	240 min P2	7.11±0.06	6.90±0.04	6.95±0.15	7.09±0.06	-
	300 min P2	7.06±0.08	-	-	7.09±0.12	-
Lactate (mmol/L)	Baseline	2.53±0.30	2.10±0.23	2.11±0.31	2.61±0.14	1.83±0.18
	60 min P1	1.63±0.11	3.05±0.75	5.20±1.34	2.84±0.28	6.83±1.74†
	60 min P2	2.14±0.21	6.33±2.89*	6.52±1.79	3.51±0.31	8.13±1.98†
	120 min P2	2.43±0.30	7.20±2.11	8.93±1.86*	4.65±0.47	10.86±2.84
	180 min P2	3.59±0.76	9.98±1.78	12.92±3.07†	6.17±1.05	10.67±1.46
	240 min P2	9.50±2.24	14.00±1.87	13.67±5.04	7.85±1.61	-
	300 min P2	10.56±2.07	-	-	9.20±6.80	-
Base Excess (mmol/L)	Baseline	-0.49±1.61	-2.78±3.09	3.90±0.68	0.97±0.83	0.76±1.64
	60 min P1	-2.39±0.62	-6.93±2.33	-8.81±2.81	-4.88±0.80	-13.26±1.97†
	60 min P2	-2.37±0.88	-11.48±3.67†	-13.08±2.91*	-6.46±0.67	-17.03±2.33†
	120 min P2	-3.05±0.91	-12.63±2.51*	-17.60±3.02†	-9.56±1.49	-23.26±1.07†
	180 min P2	-5.80±2.44	-19.78±1.16*	-19.26±4.46	-15.68±1.42	-22.80±2.06*
	240 min P2	-16.40±2.43	-28.83±1.36	-19.73±6.12	-18.03±1.46	-
	300 min P2	-15.04±2.34	-	-	-22.50±4.30	-
HCO_3^- (mmol/L)	Baseline	23.99±1.24	22.04±2.41	26.64±0.66	25.37±0.79	24.55±1.41
	60 min P1	20.84±0.62	18.68±1.74	16.71±1.89	20.02±0.60	13.53±1.52†
	60 min P2	20.76±0.84	12.80±2.98†	13.65±2.12*	18.64±0.48	11.15±1.55†
	120 min P2	20.40±0.49	14.18±1.76*	10.82±2.00†	16.32±0.92	7.28±0.78†
	180 min P2	18.74±1.53	9.48±0.92*	9.80±2.88*	12.75±0.74*	7.43±0.95*
	240 min P2	11.57±1.63	6.48±0.89	11.55±6.25	10.58±0.46	-
	300 min P2	10.62±1.50	-	-	8.70±2.70	-

* $p<0.05$ c.f. Sham
# $p<0.05$ c.f. 3% NaCl ALM
† $p<0.05$ c.f. Sham and 3% NaCl ALM
¶ $p<0.05$ c.f. Sham, No Treatment, and 3% NaCl ALM
All values are arterial.
Note: Baseline values were taken during 30 min stabilization period after surgical instrumentation but before laparotomy.

APPENDIX 2
Uncontrolled 'Non-Compressible' Hemorrhage and Shock
Electrolytes

Parameter	Time	Sham	No Treatment	3% NaCl	3% NaCl ALM	Hextend
K^+ (mmol/L)	Baseline	3.81±0.21	4.24±0.16	3.94±0.14	4.27±0.15	3.94±0.12
	60 min P1	4.36±0.07	4.35±0.29	4.60±0.34	4.01±0.12	6.31±1.71
	60 min P2	4.70±0.21	8.56±3.02	5.98±2.15	4.57±0.16	6.28±1.51
	120 min P2	5.38±0.15	5.38±0.52	7.38±2.19	5.20±0.13	12.58±3.02¶
	180 min P2	5.55±0.24	9.24±2.70	11.44±3.10	6.16±0.75	10.07±1.85
	240 min P2	11.53±2.06	17.43±1.93	13.55±7.95	10.85±2.08	-
	300 min P2	14.14±1.69	-	-	12.20±5.70	-
Na^+ (mmol/L)	Baseline	140.13±1.47	142.29±1.87	141.88±1.27	136.50±1.94	139.13±1.96
	60 min P1	140.25±0.90	142.86±3.32	141.25±1.42	143.56±1.65	134.86±1.32
	60 min P2	140.86±0.96	145.86±4.28	144.67±3.37	140.47±1.73	136.50±2.26
	120 min P2	140.50±0.96	142.00±1.82	145.60±1.89	144.18±3.04	138.00±3.58
	180 min P2	138.63±1.05	139.80±3.65	141.00±4.09	141.78±1.16	145.67±4.37
	240 min P2	134.14±2.76	135.33±1.20	132.33±6.96	147.25±7.49	-
	300 min P2	134.00±1.90	-	-	136.00±2.00	-
Ca^{2+} (mmol/L)	Baseline	1.24±0.06	1.38±0.04	1.15±0.09	1.28±0.03	1.19±0.09
	60 min P1	1.22±0.07	1.40±0.10	0.97±0.11	1.34±0.03	1.30±0.01
	60 min P2	1.32±0.02	1.42±0.12	0.99±0.11¶	1.31±0.02	1.24±0.07
	120 min P2	1.25±0.05	1.37±0.02	1.12±0.08	1.33±0.05	1.00±0.13†
	180 min P2	1.26±0.03	1.23±0.08	1.07±0.08	1.20±0.06	0.71±0.23¶
	240 min P2	1.20±0.05	1.24±0.06	1.17±0.09	1.25±0.16	-
	300 min P2	1.18±0.04	-	-	1.41±0.41	-
Cl^- (mmol/L)	Baseline	112.00±3.76	106.71±1.87	104.88±1.55	104.61±1.44	110.88±3.25
	60 min P1	109.63±0.89	110.14±4.53	103.50±0.73	111.00±1.08	106.43±1.15
	60 min P2	107.00±0.69	109.57±2.23	112.67±5.83	108.47±0.75	110.50±1.38
	120 min P2	107.00±1.41	109.00±1.23	115.00±2.49	112.18±2.25	113.40±2.87
	180 min P2	114.00±4.10	112.00±3.05	109.60±2.14	115.78±2.63	114.00±0.69
	240 min P2	114.00±6.56	115.14±2.92	109.67±0.88	104.33±2.60	-
	300 min P2	110.00±0.95	-	-	102.50±6.50	-

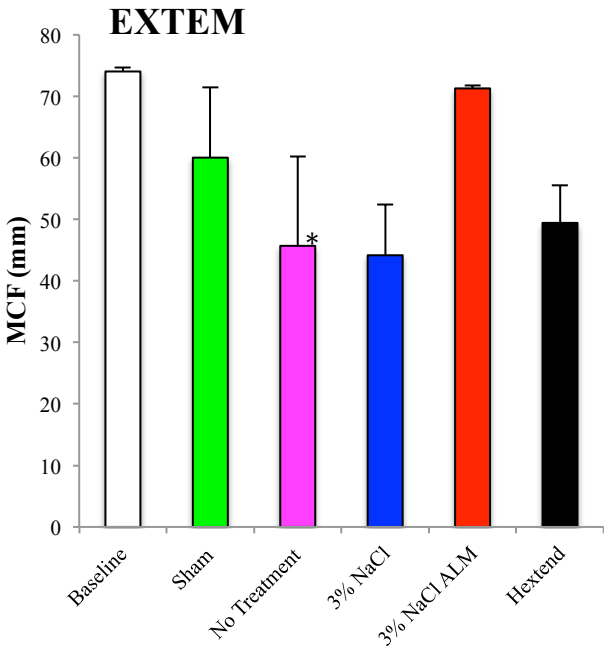
¶ $p<0.05$ c.f. Sham, No Treatment, and 3% NaCl ALM
† $p<0.05$ c.f. Sham and 3% NaCl ALM
All values are arterial.
Note: Baseline values were taken during 30 min stabilization period after surgical instrumentation but before laparotomy.

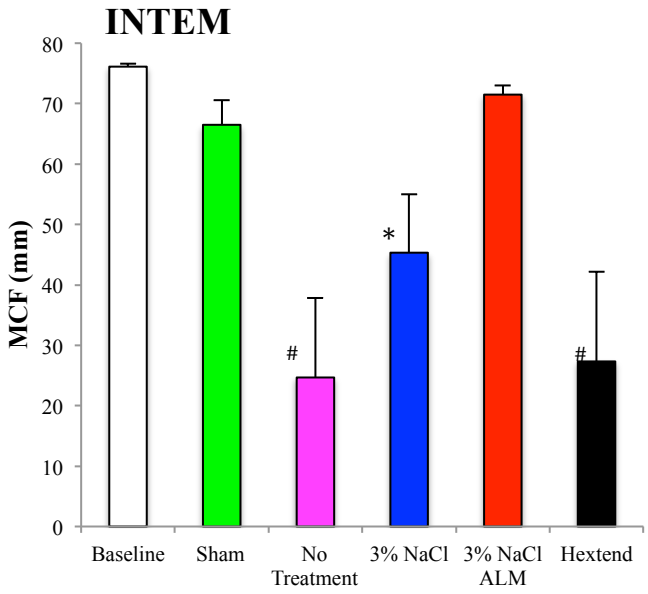
APPENDIX 3
Uncontrolled 'Non-Compressible' Hemorrhage and Shock
ROTEM Clot Initiation and Propagation Phase 1 Resuscitation

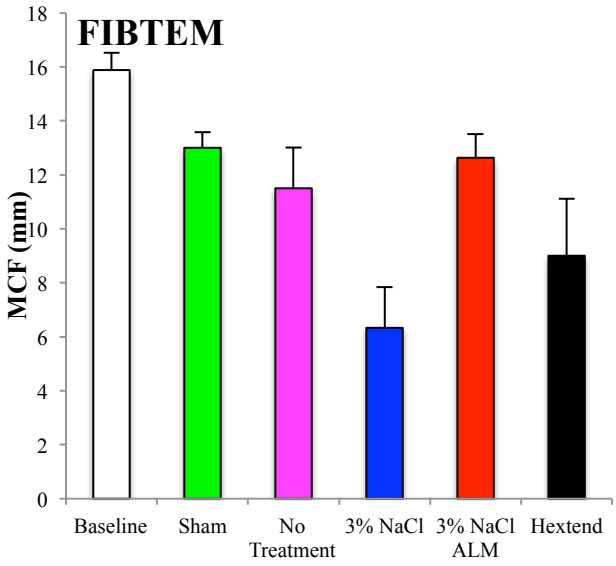
Test	Group Phase 1 Resuscitation	Clot Initiation (%)	CT (sec)	CFT (sec)	α (°)	CFR (°)	MCE	MaxV (mm/min)	MaxVt (s)	MCFt (s)	TPI
EXTEM	Baseline	100	39.88±0.92	33.63±1.15	83.25±0.25	83.88±0.23	286.13±8.47	37.50±1.12	70.63±2.52	1456.00±102.44	255.88±8.96
	Sham	75	213.67±154.54	34.40±3.67	82.80±0.74	71.00±12.61	218.33±50.76	30.83±6.38	435.17±347.61	1511.33±95.30	227.00±37.87
	No Treatment	38	161.67±89.36	44.00±4.00	59.67±21.34*	63.00±19.50*	152.50±27.50†	21.33±9.94*	182.00±81.13	1478.00±884.96	106.50±28.50
	3% NaCl	88	153.71±53.40	318.71±140.09#	57.86±10.32#	61.43±8.93*	113.86±39.53#	16.57±5.54#	228.71±76.72	1533.29±282.79	71.43±37.38
	3% NaCl ALM	100	46.38±2.63	34.63±1.05	83.00±0.19	84.00±0.19	247.75±6.61	37.88±1.16	72.13±1.70	1399.25±136.58	217.25±11.56
	Hextend	63	281.00±112.70	267.20±123.57*	57.40±11.61#	61.40±10.36*	143.33±14.77*	14.40±5.41#	394.60±171.56	1717.40±522.56	75.67±22.60
INTEM	Baseline	100	92.38±5.24	27.25±1.19	84.50±0.19	85.63±0.18	319.63±7.79	50.00±1.57	114.63±6.25	1327.88±47.12	356.13±19.94
	Sham	50	461.75±298.99†	241.00±185.21	64.75±14.02†	68.00±12.09†	237.67±41.80	20.00±6.87†	689.75±483.90†	1902.75±121.40	150.33±51.17†
	No Treatment	38	458.33±145.54†	169 (n=1)	25.67±17.68*	34.67±17.19*	59.50±50.76	4.00±3.00*	564.00±152.16*	2064.33±620.94	19 (n=1)
	3% NaCl	75	349.33±226.76	319.60±130.43	46.00±11.36*	56.00±8.21*	186.67±97.16	11.83±6.19*	446.17±286.43	2478.50±323.47†	184.00±1.33
	3% NaCl ALM	100	255.25±72.84†	58.00±16.18	78.88±2.84	80.50±2.29	257.75±16.49	31.75±5.20†	302.25±88.33†	1706.38±54.81	200.50±41.70†
	Hextend	38	680.33±250.83†	50 (n=1)	45.00±35.00†	35.33±22.88†	134 (n-1)	9.00±8.00†	957.67±406.19†	1975.00±653.18	80 (n=1)
FIBTEM	Baseline	100	36.13±0.61	-	78.88±1.06	79.63±1.02	18.88±0.77	23.38±2.04	40.13±0.88	522.75±167.23	-
	Sham	75	587.17±525.45†	-	71.50±2.33	59.20±13.90	13.80±1.28	9.38±2.70	626.83±560.91	482.83±217.57	-
	No Treatment	25	86.00±30.00*	-	59 (n=1)	61 (n=1)	13.00±2.00	4.50±2.50	129.00±0 (n=2)	1590.00±319.00	-
	3% NaCl	75	324.67±162.22*	-	75 (n=1)	76.00±1.00	7.00±1.79	6.80±3.58	193.00±88.01	847.50±161.78	-
	3% NaCl ALM	100	45.13±2.41	-	74.63±0.82	75.88±0.77	14.50±1.20	16.25±0.90	49.13±2.82	157.88±27.59	-
	Hextend	50	399.50±217.90*	-	68 (n=1)	71 (n=1)	9.00±3.22	4.75±2.50	340.20±191.73	1326.00±279.62	-
APTEM	Baseline	100	42.63±1.43	33.00±1.05	83.50±0.27	83.88±0.13	275.13±6.52	38.00±0.85	73.88±2.54	1444.50±89.39	251.13±10.02
	Sham	63	82.60±23.06	46.80±11.86	80.60±2.42	81.80±1.72	230.00±7.82†	33.00±4.70	116.40±30.73	1319.80±136.92	173.80±27.80†
	No Treatment	25	154.50±57.50	51 (n=1)	52.50±27.50*	54.50±27.50	165 (n=1)	14.50±12.50‡	185.50±50.50	1930.50±1380.50	96 (n=1)
	3% NaCl	75	236.33±102.86	71.75±23.73	63.00±13.47*	56.50±14.09	103.13±42.60*	17.00±6.47*	341.17±140.38	1219.83±256.45	100.00±50.06†
	3% NaCl ALM	100	50.75±2.39	33.38±1.48	83.13±0.30	84.00±0.27	232.88±7.48†	39.13±1.76	76.88±2.91	1309.00±113.37	212.75±14.72
	Hextend	63	388.20±163.03*	402.60±199.53	52.20±13.32#	55.80±12.19	135.00±5.86#	13.00±5.44#	579.00±286.02	1976.20±526.28	64.67±22.15*

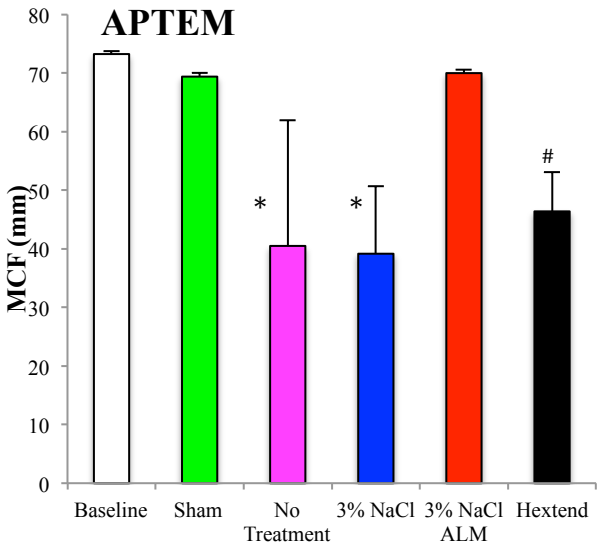
* $p<0.05$ c.f. Baseline and 3% NaCl ALM
# $p<0.05$ c.f. Baseline, Sham, and 3% NaCl ALM
† $p<0.05$ c.f. Baseline
‡ $p<0.05$ c.f. 3% NaCl ALM

APPENDIX 4
Uncontrolled 'Non-Compressible' Hemorrhage and Shock
ROTEM Clot Strength Phase 1 Resuscitation









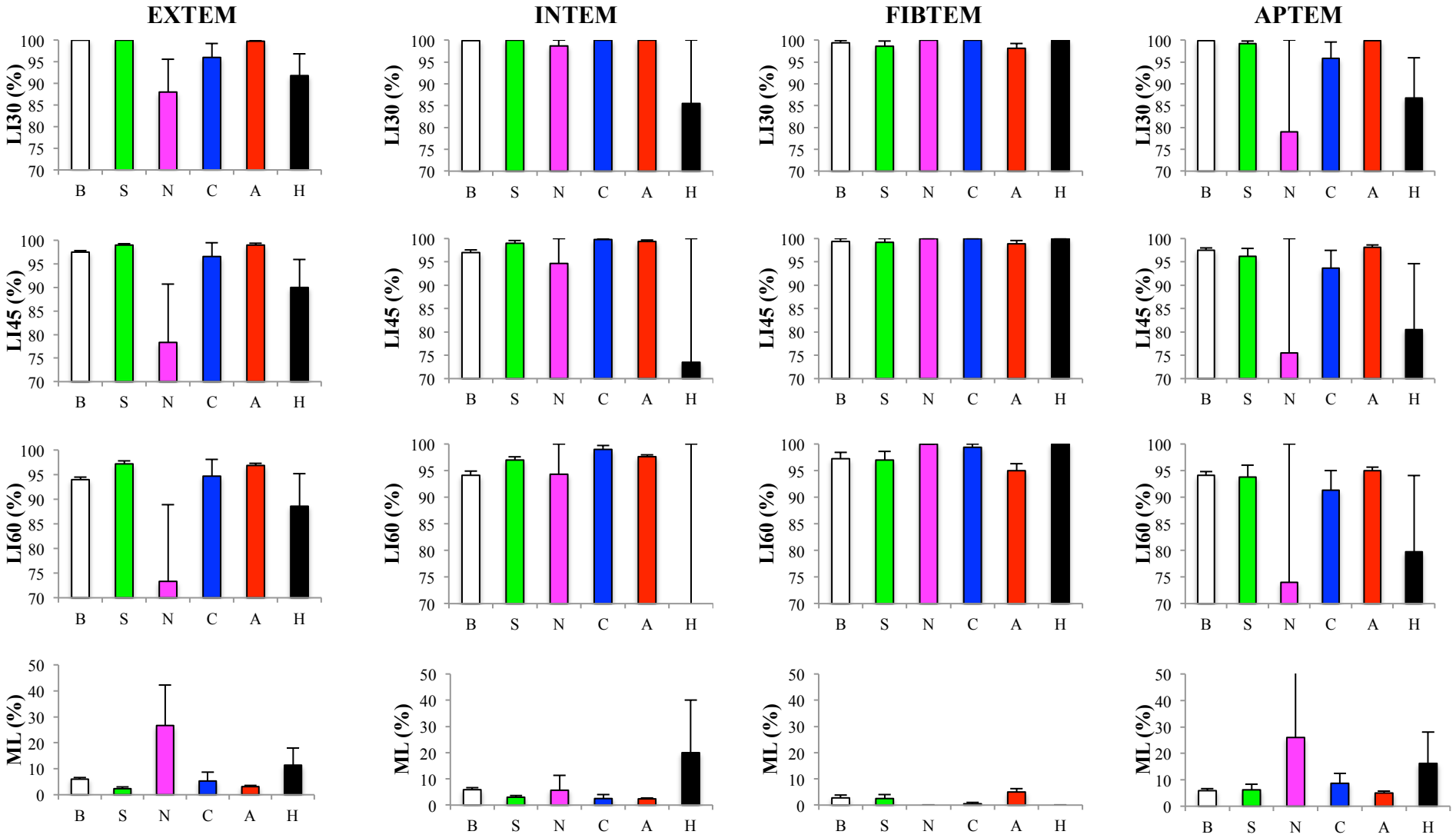
APPENDIX 5
Uncontrolled 'Non-Compressible' Hemorrhage and Shock
ROTEM Clot Lysis Phase 1 Resuscitation

Test	Lysis Parameter	Baseline	Sham	No Treatment	3% NaCl	3% NaCl ALM	Hextend
EXTEM	Lysis Initiation (%)	0 (0/8)	0 (0/6)	67 (2/3)	14 (1/7)	0 (0/8)	40 (2/5)
	Lysis Onset Time (sec)	-	-	1569±607	280 (n=1)	-	1479±294
	Clot Lysis Rate (°)	-	-	45 (n=1)	24 (n=1)	-	41±4
INTEM	Lysis Initiation (%)	0 (0/8)	0 (0/4)	33 (1/3)	0 (0/6)	0 (0/8)	33 (1/3)
	Lysis Onset Time (sec)	-	-	2567 (n=1)	-	-	1076 (n=1)
	Clot Lysis Rate (°)	-	-	-	-	-	-
FIBTEM	Lysis Initiation (%)	0 (0/8)	0 (0/6)	0 (0/2)	0 (0/6)	0 (0/8)	0 (0/4)
	Lysis Onset Time (sec)	-	-	-	-	-	-
	Clot Lysis Rate (°)	-	-	-	-	-	-
APTEM	Lysis Initiation (%)	0 (0/8)	0 (0/5)	50 (1/2)	20 (1/5)	0 (0/8)	40 (2/5)
	Lysis Onset Time (sec)	-	-	1052 (n=1)	293 (n=1)	-	1083±442
	Clot Lysis Rate (°)	-	-	53 (n=1)	25 (n=1)	-	16 (n=1)

Lysis indices at 30 min, 45 min, and 60 min (LI30-60, %), as well as maximum lysis (ML, %) for EXTEM, INTEM, FIBTEM, and APTEM test are shown on the next page. B=baseline, S=sham, N=no treatment, C=3% NaCl, A=3% NaCl ALM, and H=Hextend. * $p<0.05$ compared to all groups except Hextend; $^{\#}$ $p<0.05$ compared to all groups except No Treatment. Number of measurements for each calculated lysis parameter are Baseline (8), Sham (6), No Treatment (3 for EXTEM, INTEM and APTEM; 2 for FIBTEM), 3% NaCl (6), 3% NaCl ALM (8), and Hextend (5 for EXTEM and APTEM; 4 for FIBTEM; and 3 for INTEM).

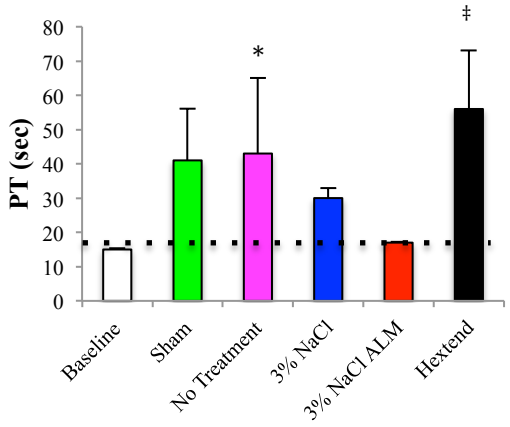
APPENDIX 6

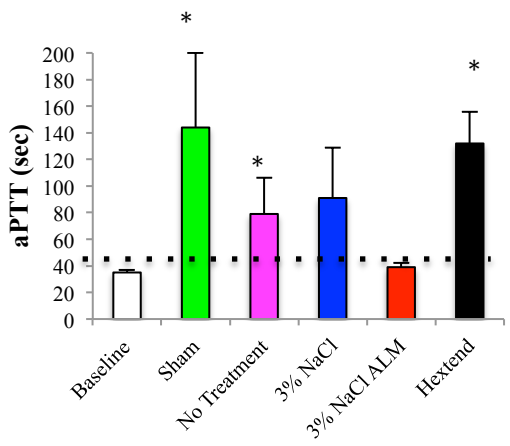
Uncontrolled 'Non-Compressible' Hemorrhage and Shock ROTEM Clot Lysis Phase 1 Resuscitation

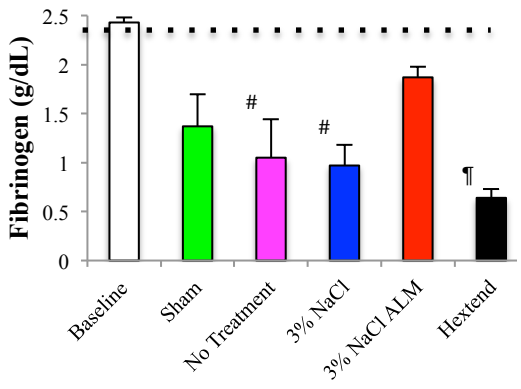


APPENDIX 7
Uncontrolled 'Non-Compressible" Hemorrhage and Shock
Prothrombin Time (PT), Activated Partial Thromboplastin Time (aPTT), Fibrinogen (g/dL) (Diagnostica Stago STA Compact)

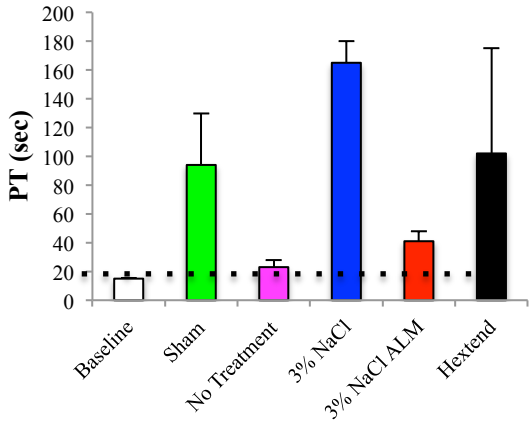
PHASE 1 RESUSCITATION

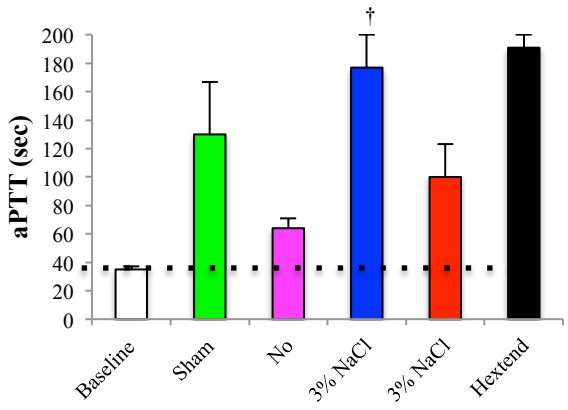


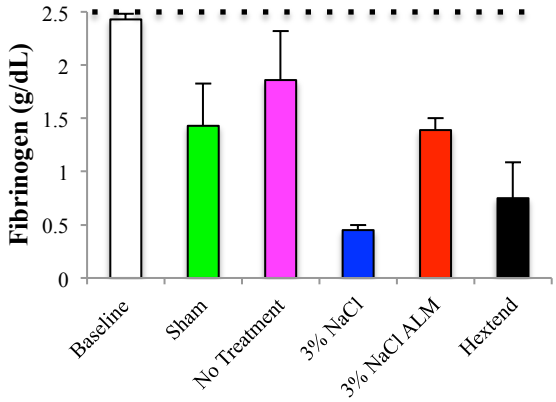




PHASE 2 RESUSCITATION







* $p<0.05$ c.f. Baseline and 3% NaCl ALM
[#] $p<0.05$ c.f. Baseline
[†] $p<0.05$ c.f. 3% NaCl ALM
[¶] $p<0.05$ c.f. Baseline, Sham, 3% NaCl ALM
[‡] $p<0.05$ c.f. all except No Treatment

Phase 2 Resuscitation n=2 for Hextend group, n=3 for No Treatment group, and n=5 for 3% NaCl group

APPENDIX 8
Uncontrolled 'Non-Compressible" Hemorrhage and Shock
Platelet Function

Platelet function was assessed in platelet rich plasma (PRP) using the PAP-8e Platelet Aggregation Profiler and agonists ADP (BioData adenosine-5'-diphosphate reagent, 2 x 10^{-4}) and Collagen (BioData soluble calf skin collagen, 1.9 mg/ml). PRP was prepared using a standardized technique of double centrifugation (Messora *et al*., 2011).

ADP-Induced Aggregation	**Baseline**	**Sham**	**No Treatment**	**3% NaCl**	**3% NaCl ALM**	**Hextend**
Primary Aggregation (%)	103.13† (8.00)	13.63 (6.09)	0.78* (0.23)	0.75* (0.49)	32.63 (13.71)	4.00$^{\#}$ (1.35)
Primary Slope	85.38† (12.23)	10.13 (6.00)	0.00* (0.00)	0.00* (0.00)	35.25 (18.66)	2.13$^{\#}$ (1.39)
Disaggregation (%)	2.38 (1.49)	7.63 (5.43)	0.00 (0.00)	0.00 (0.00)	19.00 (14.54)	0.00 (0.00)
Final Aggregation (%)	95.13† (12.08)	8.13 (6.00)	0.00* (0.00)	0.50* (0.50)	18.00 (7.73)	3.38$^{\#}$ (1.18)
Collagen-Induced Aggregation	**Baseline**	**Sham**	**No Treatment**	**3% NaCl**	**3% NaCl ALM**	**Hextend**
Primary Aggregation (%)	115.50† (3.50)	32.63 (19.20)	0.89* (0.31)	1.50* (0.38)	14.13 (4.48)	6.00$^{\#}$ (1.77)
Primary Slope	99.75† (13.90)	44.13 (26.88)	0.00* (0.00)	0.00* (0.00)	13.63 (5.82)	3.13$^{\#}$ (1.39)
Disaggregation (%)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	9.88 (3.62)	1.25 (0.82)
Final Aggregation (%)	99.63† (12.95)	31.50 (18.79)	0.00$^{\#}$ (0.00)	0.00$^{\#}$ (0.00)	4.00 (1.63)	3.75 (1.52)

Data represents mean with standard error of the mean in parentheses
* $p<0.05$ c.f. Sham and 3% NaCl ALM
$^{\#}$ $p<0.05$ c.f. 3% NaCl ALM
† $p<0.05$ c.f. all groups

APPENDIX 9
Uncontrolled 'Non-Compressible' Hemorrhage and Shock: Systemic (Plasma) Inflammation

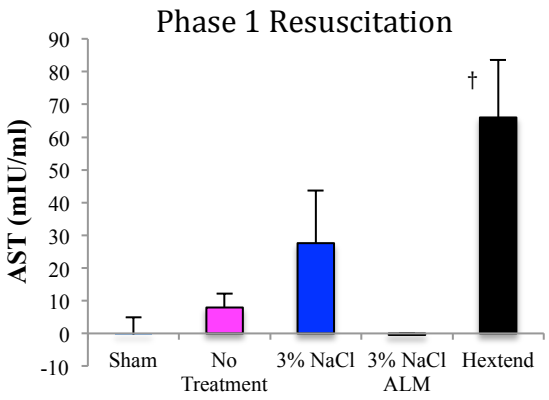
GM-CSF, IFN-γ, IL-10, IL-12 (p70), IL-1α, IL-1β, IL-2, IL-4, IL-8, RANTES, and TNF-α were measured in plasma after Phase 1 (60 min) resuscitation and Phase 2 (300 min or death) resuscitation using Milliplex Rat Cytokine Panel according to manufacturer's instructions.

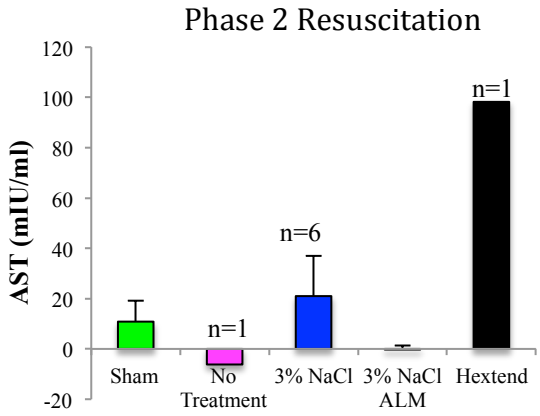
Group	Resuscitation Phase	GM-CSF (pg/ml)	IFN-γ (pg/ml)	IL-10 (pg/ml)	IL-12 p70 (pg/ml)	IL-1α (pg/ml)	IL-1β (pg/ml)	IL-2 (pg/ml)	IL-4 (pg/ml)	IL-6 (pg/ml)	RANTES (pg/ml)	TNF-α (pg/ml)
Sham	Phase 1	35.08 (3.85)	6.88 (2.99)	2939.83 (829.45)	44.47 (10.97)	2.10 (1.72)	0.06 (0.03)	3.77 (0.98)	0.19 (0.17)	194.75 (62.36)	377.92 (38.52)	40.46 (11.17)
	Phase 2	36.67 (3.84)	43.87 (19.76)	377.09 (82.15)	32.67 (7.42)	123.62 (55.29)	188.31 (69.55)	22.76 (2.78)	10.47 (3.61)	21197.92 (9481.57)	1375.31 (351.14)	10.33 (1.46)
No Treatment	Phase 1	37.60 (4.36)	19.50[#] (5.45)	1270.75[†] (371.70)	25.73 (3.78)	261.97* (103.60)	114.51* (63.07)	70.82 (58.64)	6.76 (4.03)	3731.60* (2136.54)	1236.01 (389.76)	19.37 (10.37)
	Phase 2 (n=1)	29.52	20.55	1753.00	32.03	13.86	27.48	33.89	-	19273.00	698.83	22.31
3% NaCl	Phase 1	34.63 (9.66)	18.30 (10.94)	2811.04 (556.45)	34.59 (6.30	205.15 (131.54)	49.67 (38.33)	8.08[#] (2.88)	Not detected	1323.85[#] (871.03)	868.70* (308.17)	45.61 (11.44)
	Phase 2 (n=6)	42.97 (2.95)	208.11[#] (99.58)	2017.00* (335.40)	27.39 (3.01)	444.92* (84.37)	617.96* (84.62)	27.13[#] (4.79)	12.46 (10.19)	110930.50[#] (21244.93)	1817.85* (312.45)	33.36* (5.86)
3% NaCl ALM	Phase 1	33.75 (6.64)	2.76 (1.66)	1819.84 (453.12)	24.04 (7.76)	Not detected	Not detected	1.87 (1.48)	Not detected	82.07 (27.39)	297.70 (85.96)	11.26 (3.89)
	Phase 2	35.45 (5.15)	11.46[£] (3.05)	196.82 (81.79)	32.95 (6.83)	22.02 (19.18)	32.20[£] (21.68)	8.19[£] (2.32)	Not detected	9142.31 (3999.69)	637.54 (139.70)	4.11[£] (1.25)
Hextend	Phase 1	56.08[¶] (7.31)	21.92[#] (7.18)	277.38[‡] (140.78)	20.11[†] (2.22)	297.34* (65.07)	165.80* (66.78)	21.73[#] (8.45)	10.54* (4.82)	633.40* (136.00)	1098.65* (206.05)	14.93 (9.06)
	Phase 2 (n=1)	41.82	255.18	4259.00	20.93	884.97	829.98	61.91	Not detected	172100.00	3318.00	37.68

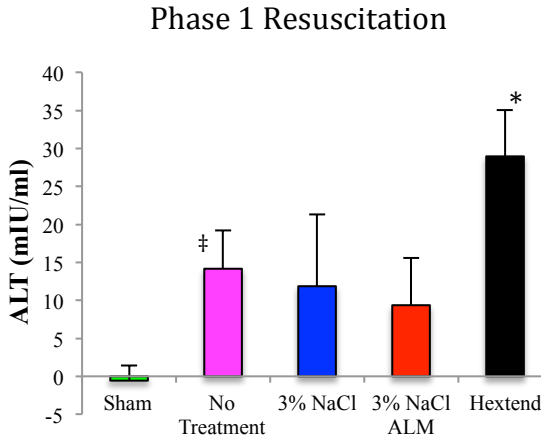
Data represent mean with standard error of the mean in parentheses
n=8 unless indicated
* $p<0.05$ c.f. Sham and 3% NaCl ALM
[#] $p<0.05$ c.f. 3% NaCl ALM
[£] $p<0.05$ c.f. Sham
[†] $p<0.05$ c.f. 3% NaCl
[‡] $p<0.05$ c.f. 3% NaCl and No Treatment
[¶] $p<0.05$ c.f. all other groups

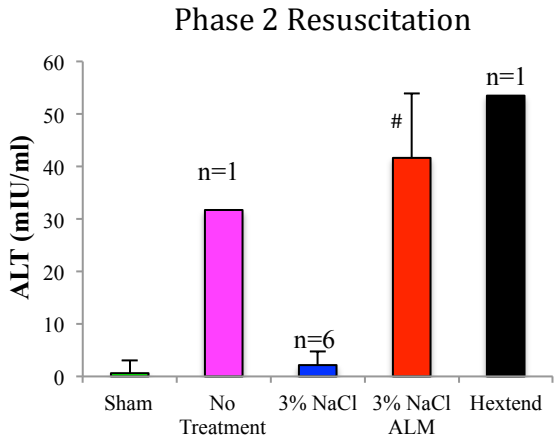
APPENDIX 10
Organ Injury Markers: AST, ALT and NAG

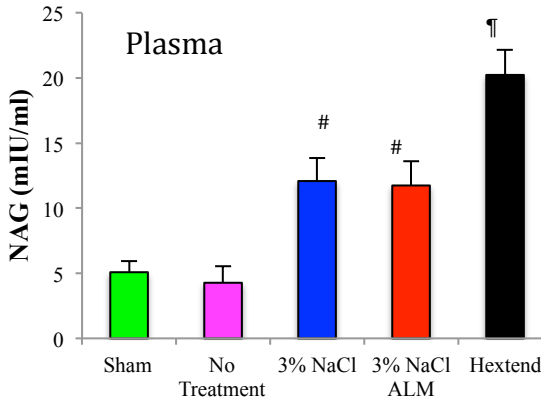
Plasma levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), and plasma and kidney levels of N-acetyl-beta-D-glucosaminidase (NAG) were measured using rat-specific ELISA kits (Cusabio Life Science). All assays were carried out according to manufacturer's instructions.

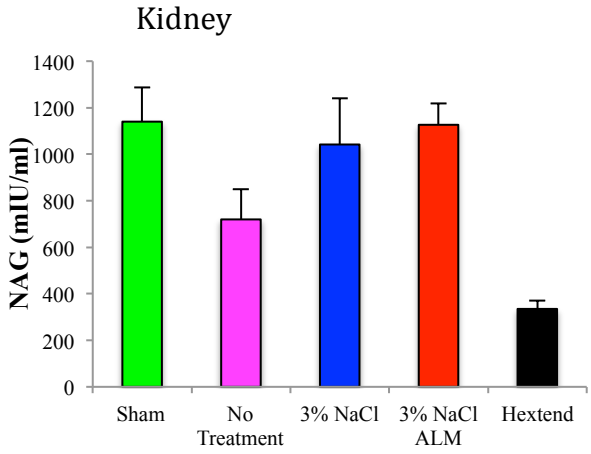








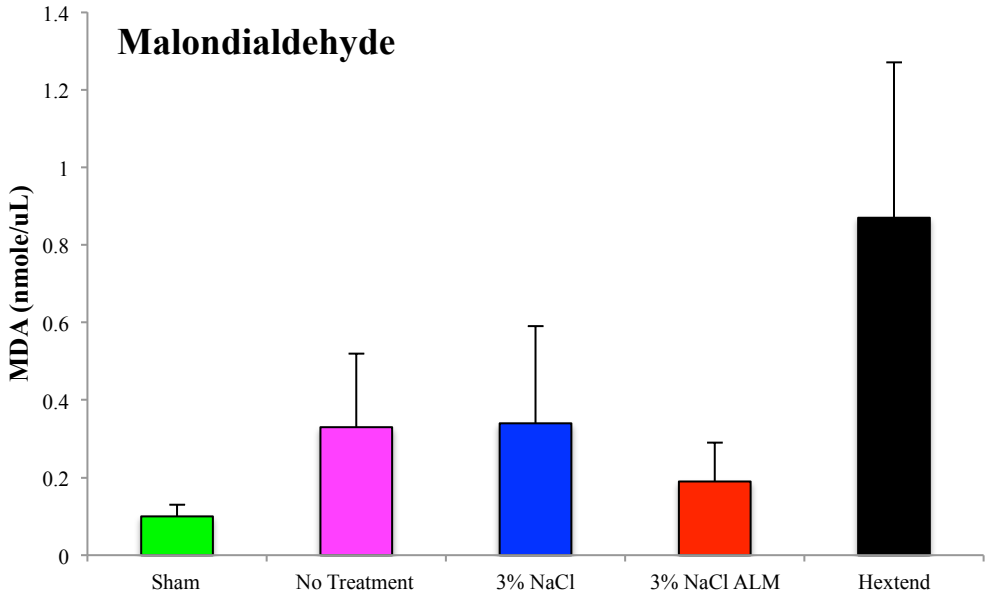




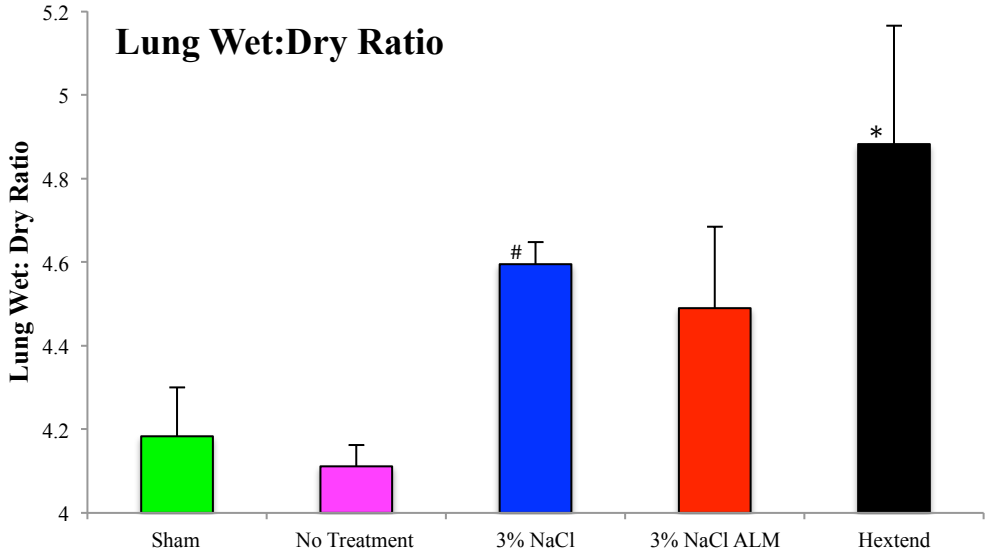
* $p<0.05$ c.f. Sham, ALM
[#] $p<0.05$ c.f. Sham, NT
[†] $p<0.05$ c.f. Sham, NT, ALM
[‡] $p<0.05$ c.f. ALM
[¶] $p<0.05$ c.f. all groups

APPENDIX 11
Uncontrolled 'Non-Compressible" Hemorrhage and Shock
Oxidative Stress (MDA) and Lung Wet-Dry Ratio

Malondialdehyde (MDA) concentration in lung tissue was measured as an indicator of oxidative stress using Lipid Peroxidation (MDA) Assay Kit (Sigma Aldrich). In this kit, lipid peroxidation is determined by the reaction of MDA with thiobarbituric acid (TBA) to form a colorimetric product (532 nm), proportional to the MDA present.



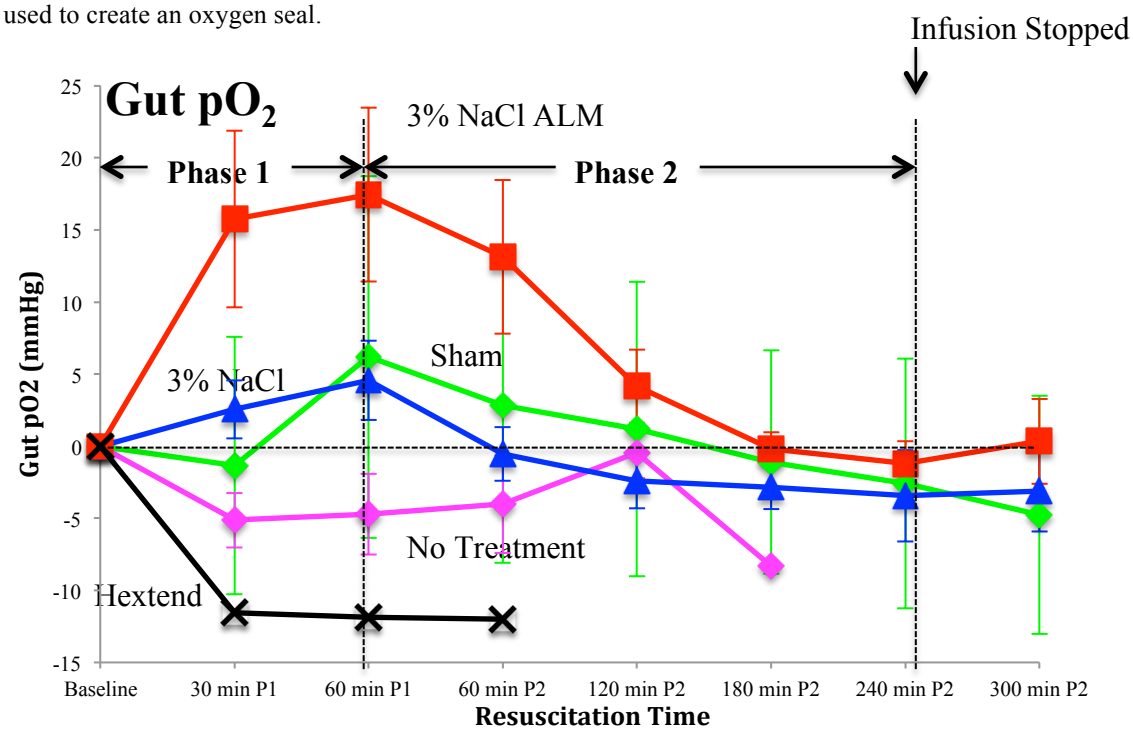
The lung wet-to-dry ratio (W/D) was measured as an index of lung water accumulation. Left lung lobe weight was measured immediately after excision (wet weight). Lung tissue was then dried in an oven at 70°C for 48 hr and re-weighed as the dry weight. The W/D weight ratio was then calculated by dividing wet by dry weight.



* $p<0.05$ c.f. Sham and No Treatment
# $p<0.05$ c.f. No Treatment

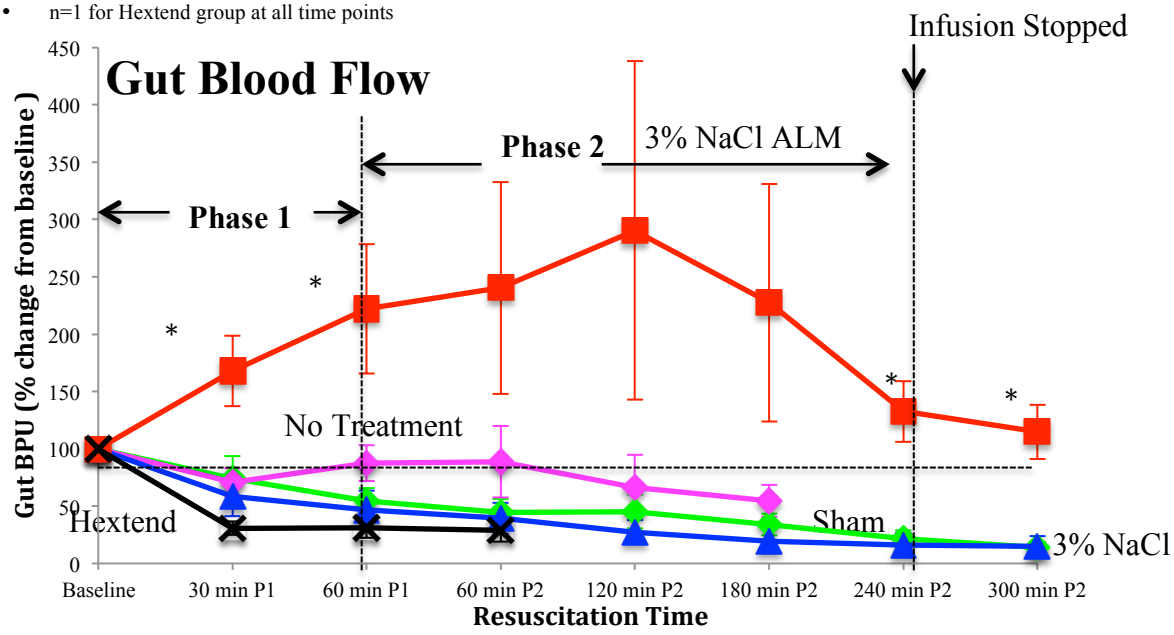
APPENDIX 12
Gut pO2 (mmHg) and Flow (BPU)

Gut tPO_2 (mmHg) and flow (BPU) was measured at the same location in the small intestine at the level of the jejunum with the Oxford Optronix pO2/Flow 'Bare-Fibre" sensor connected to Oxylite Pro XL and Oxyflo Pro recording equipment. The sensor was inserted into the mesentery with a purse stitch used to create an oxygen seal.



Data represents change in Gut pO_2 (mmHg) from Baseline values set to 0 mmHg

- n=2 for No Treatment at 60 min, 120 min, and 180 min Phase 2 resuscitation; and for 3% NaCl at 240 min and 300 min Phase 2 resuscitation
- n=1 for Hextend group at all time points



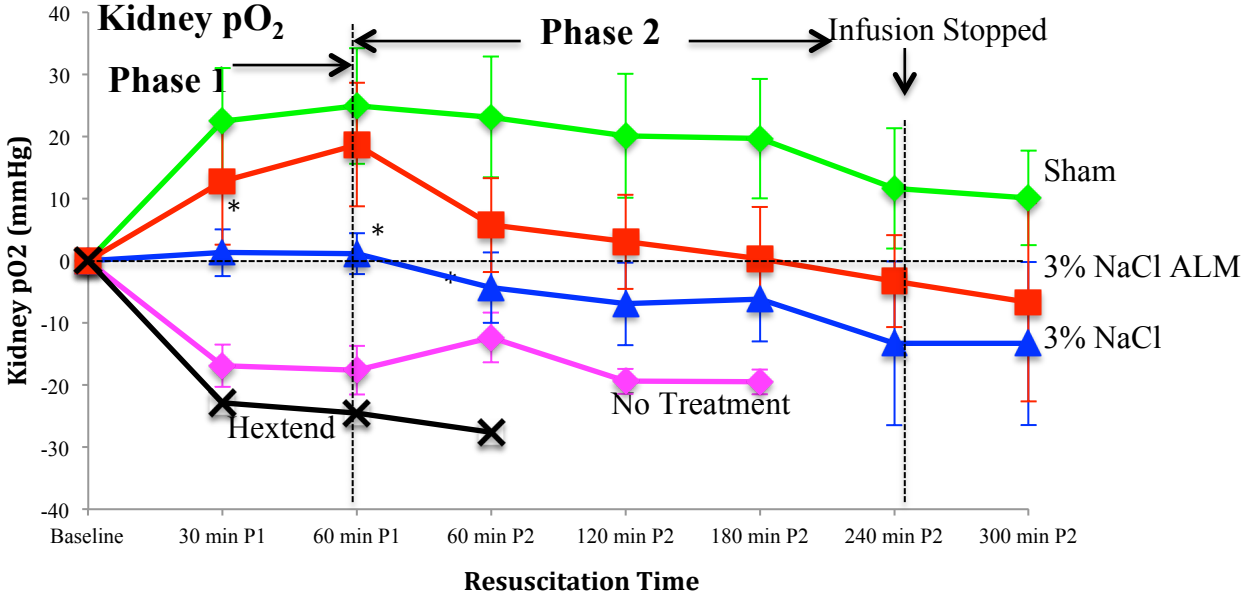
* $p<0.05$ compared to all other groups

Data represent percentage change of Gut Blood Perfusion Units (BPU) from 100% baseline

- n=2 for No Treatment at 60 min, 120 min, and 180 min Phase 2 resuscitation; and for 3% NaCl at 240 min and 300 min Phase 2 resuscitation

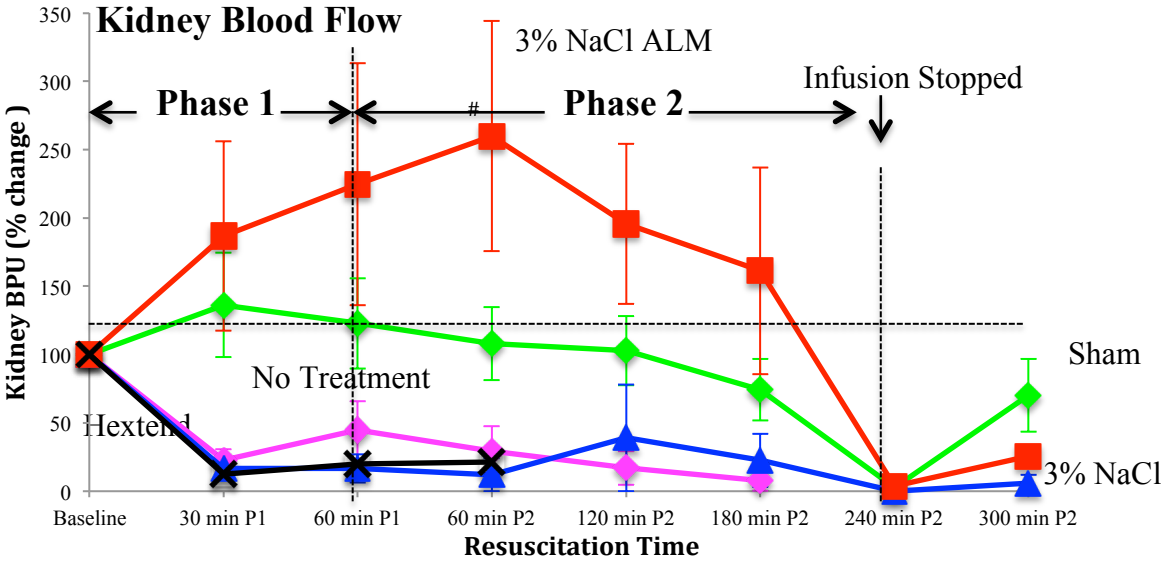
APPENDIX 13
Kidney pO2 (mmHg) and Flow (BPU)

Kidney tPO_2 (mmHg) and flow (BPU) was measured in the left kidney with the Oxford Optronix pO2/Flow 'Bare-Fibre" sensor connected to Oxylite Pro XL and Oxyflo Pro XL for data recording. A 25G guidance cannula was used to insert the sensor to a depth of 2-3 mm initially before retraction to 1-2 mm to enable monitoring at approximately the cortico-medullary junction.



* $p<0.05$ compared to Sham

- n=2 for 3% NaCl at 240 min and 300 min Phase 2 Resuscitation

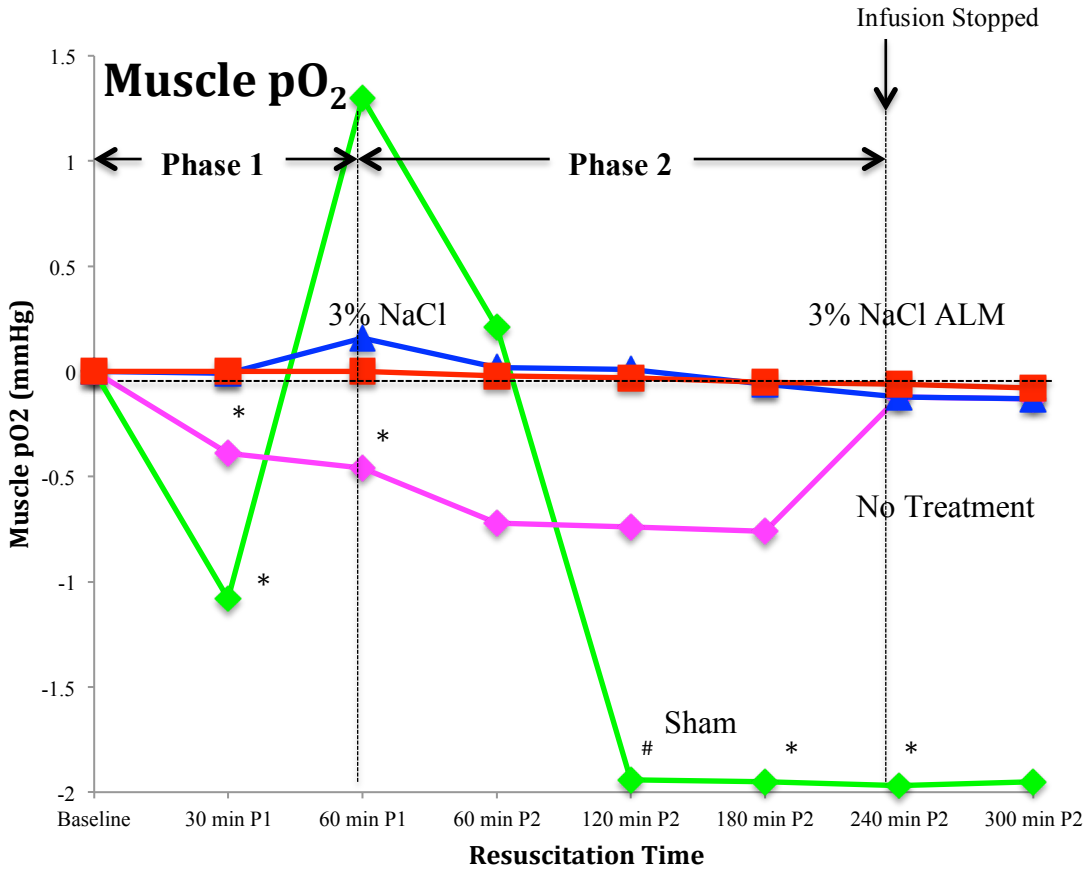


# $p<0.05$ compared to 3% NaCl

- n=2 for 3% NaCl for 240 min and 300 min Phase 2 Resuscitation

APPENDIX 14
Muscle pO2 (mmHg) and Flow (BPU)

Muscle tPO_2 (mmHg) was measured in the left vastus intermedius muscle with the Oxford Optronix pO2/Flow 'Bare-Fibre" sensor connected to Oxylite Pro XL and Oxyflo Pro XL for recording. An 18G guidance cannula was used to insert the sensor longitudinally to a depth of 10 mm.



Data represents change in Muscle tPO_2 (mmHg) from baseline set to 0 mmHg

* $p<0.05$ compared to 3% NaCl ALM
# $p<0.05$ compared to 3% NaCl and 3% NaCl ALM

- Missing values precluded Hextend group from analysis
- n=2 for No Treatment group at 120 and 180 min Phase 2 resuscitation
- n=2 for 3% NaCl group at 240 and 300 min Phase 2 resuscitation

Mean Muscle blood flow (blood perfusion units; BPU) across all groups at baseline was 65±28 BPU. Following laparotomy (Sham) or laparotomy + uncontrolled hemorrhage (No Treatment, 3% NaCl, 3% NaCl ALM) BPU decreased to undetectable levels (<0 BPU). Muscle blood flow remained <0 BPU for the remainder of the resuscitation and monitoring periods for all animals in all groups.

A New Ultra-Small Volume Fluid for Far-Forward, Non-Compressible Hemorrhage and Traumatic Brain Injury SO13004 under Award No. W81XWH-15-1-0002

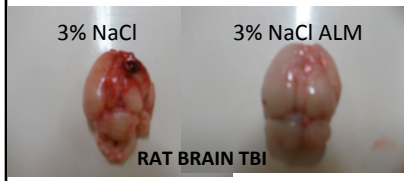
Project Description

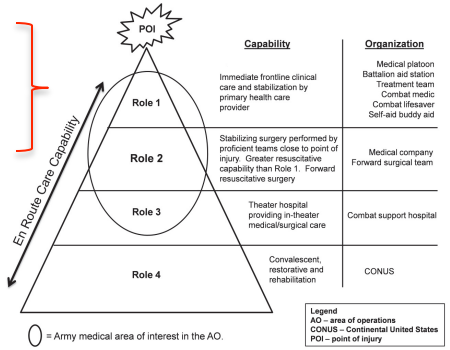
- To examine 3% NaCl ALM IV bolus (1 ml kg^{-1}) and 0.9% NaCl ALM 'drip' (1ml/kg/hr) in three rat models of: 1) hepatic hemorrhage (60% resection) and shock, 2) mild-to-moderate TBI, and 3) combined TBI and hepatic hemorrhage.

ALM Resuscitation for Point-Of-Injury (Role 1) Combat Casualty Care

Three Major DoD Capability Gaps

- Uncontrolled truncal bleeding
- Traumatic Brain Injury (TBI)
- Combined TBI and truncal bleeding





Technical Performance

Ares of Interest: POI Truncal Hemorrhage control, Shock and TBI

- **Goal**: To develop a new ultra-small-volume ALM fluid therapy for 60 min hypotensive field resuscitation and stabilization 'drip' for uncontrolled blood loss and shock, with and without suspected TBI.
- Rats were anesthetized, placed on a ventilator and instrumented. A laparotomy was performed and the liver was resected (60%) and allowed to bleed. After 15 min, a bolus of 3% NaCl ALM was injected and function monitored 60 min (Phase 1). After 60 min 0.9% NaCl ALM 'drip' was started and continued for 4 hours (Phase 2). TBI was induced by fluid-percussion (Dragonfly). Coagulation and fibrinolysis were assessed using PT, aPTT, ROTEM, STAGO and inflammation from ELIZA kits. Separate groups (double laparotomy) were required for gut, kidney and muscle pO_2 and flow (Optronix, Oxford). Cardiac function was assessed using echocardiography
- **Benefit to Special Forces Operations**: Small-volume ALM resuscitation therapy may provide SOF combat medic/corpsman with a new way to improve warfighter survivability in far-forward environments.

Cost ($K)

YEAR 1	YEAR 2	Total
$556K (2015)	$0K	$556 K

Grant Manager: Benjamin Chitty USSOCOM, SORDAC S&T
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Major Findings (Feb 29, 2016)

- In the first model, we report that the ALM one-two therapy reduced uncontrolled blood loss by up to 60% and improved blood flow to the gut and kidney. In contrast, Hextend administered according to TCCC guidelines promoted internal bleeding, acute hypocoagulopathy, inflammation, organ failure and early death.
- ALM's ability to significantly reduce blood loss may arise from its unique ability to improve cardiac function, correct coagulopathy, blunt systemic inflammation and improve tissue oxygenation.
- In the second study, ALM treatment protected against secondary brain injury following mild-to-moderate TBI from similar protective strategies.
- In the third lethal model of TBI and hemorrhage, ALM treatment resulted in 50% survivability and 50% less blood loss compared to 100% mortality in 3% saline controls.

Deliverables:

Three abstracts have been submitted for consideration at 2016 MHSRS and three manuscripts are being written for international trauma journals. A methods MS describing the TBI/hemorrhage model will also be published.